JC99 PATENT & TRADEMARK OFFICE AUG 16 2004

Appl. No. 10/792,035; Filed: March 4, 2004
Dkt No. 0942.5340005/BJD; Group Unit: 1645
Inventors: CHESNUT *et al.*; Tel: 202-371-2600
For: Methods and Compositions for Synthesis of Nucleic Acid Molecules Using Multiple Recognition Sites

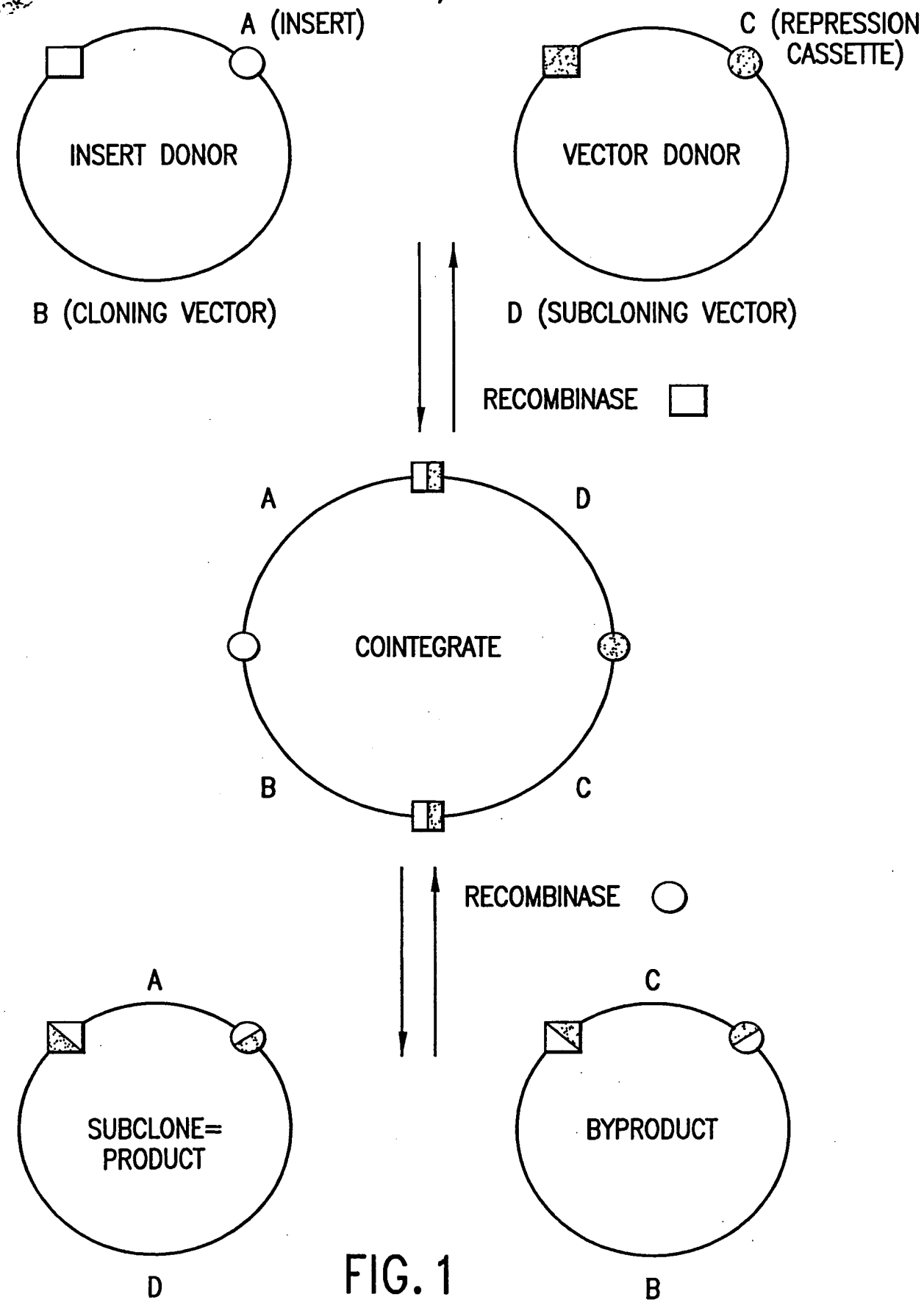


FIG. 1

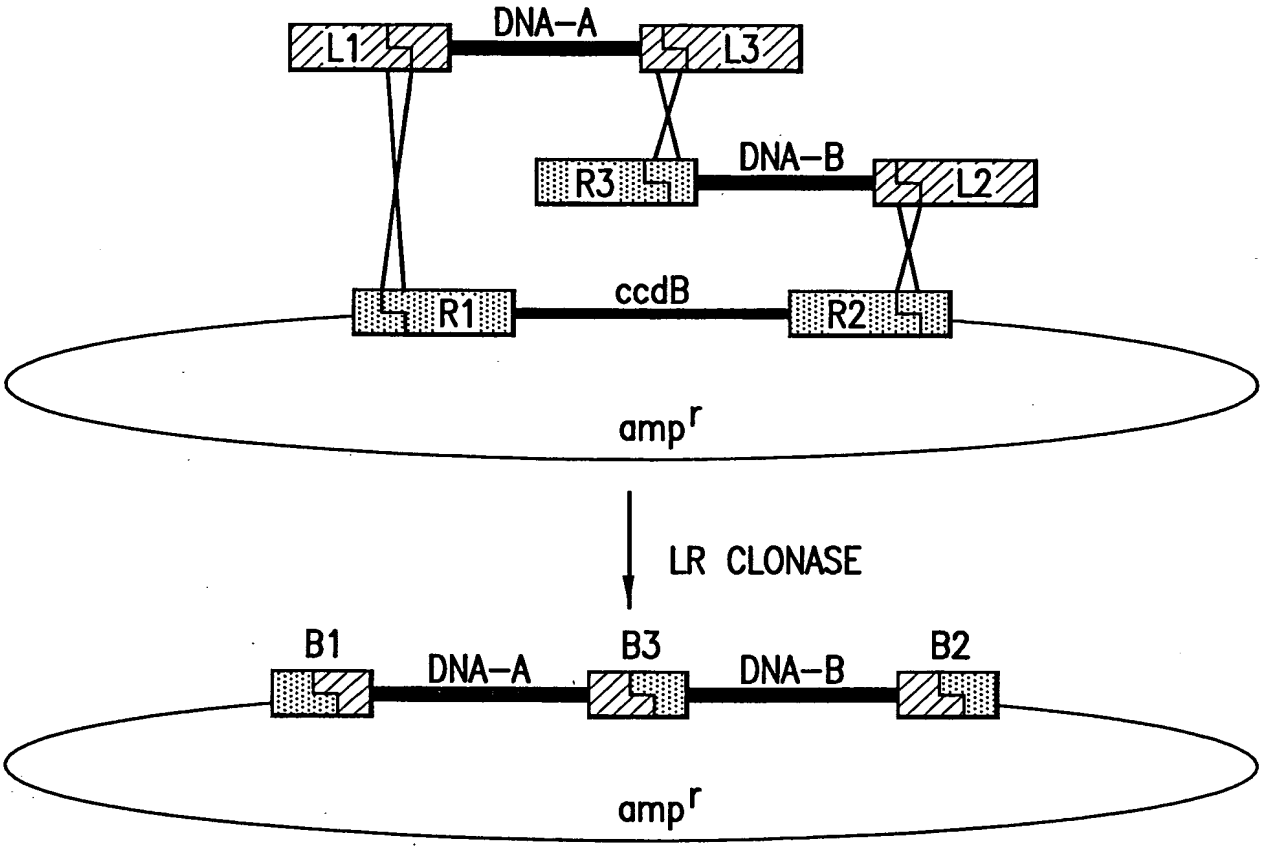

DNA–A
L1
L3
DNA–B
R3
L2
ccdB
R1
R2
amp^r
LR CLONASE
B1
DNA–A
B3
DNA–B
B2
amp^r

FIG. 2

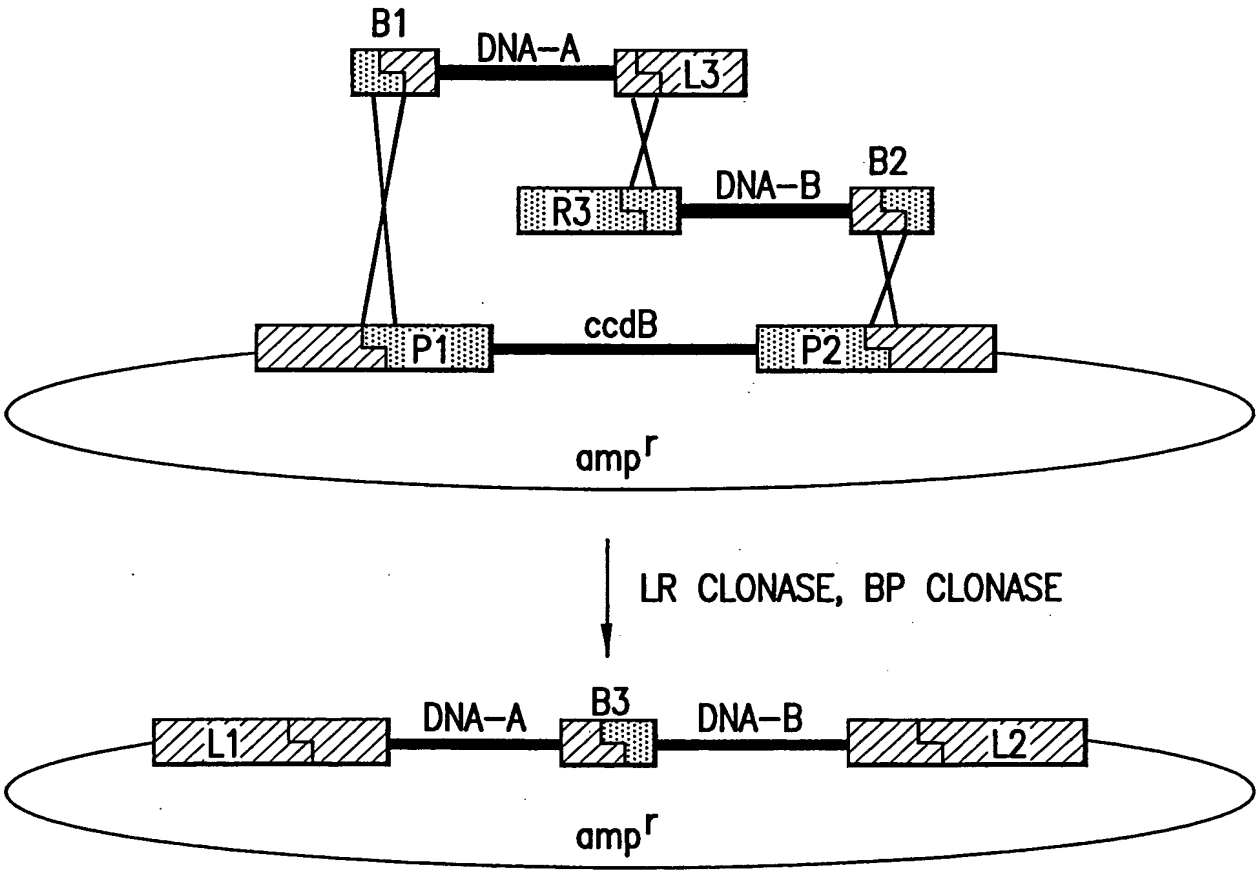

B1
DNA–A
L3
R3
DNA–B
B2
P1
ccdB
P2
amp^r
LR CLONASE, BP CLONASE
L1
DNA–A
B3
DNA–B
L2
amp^r

FIG. 3

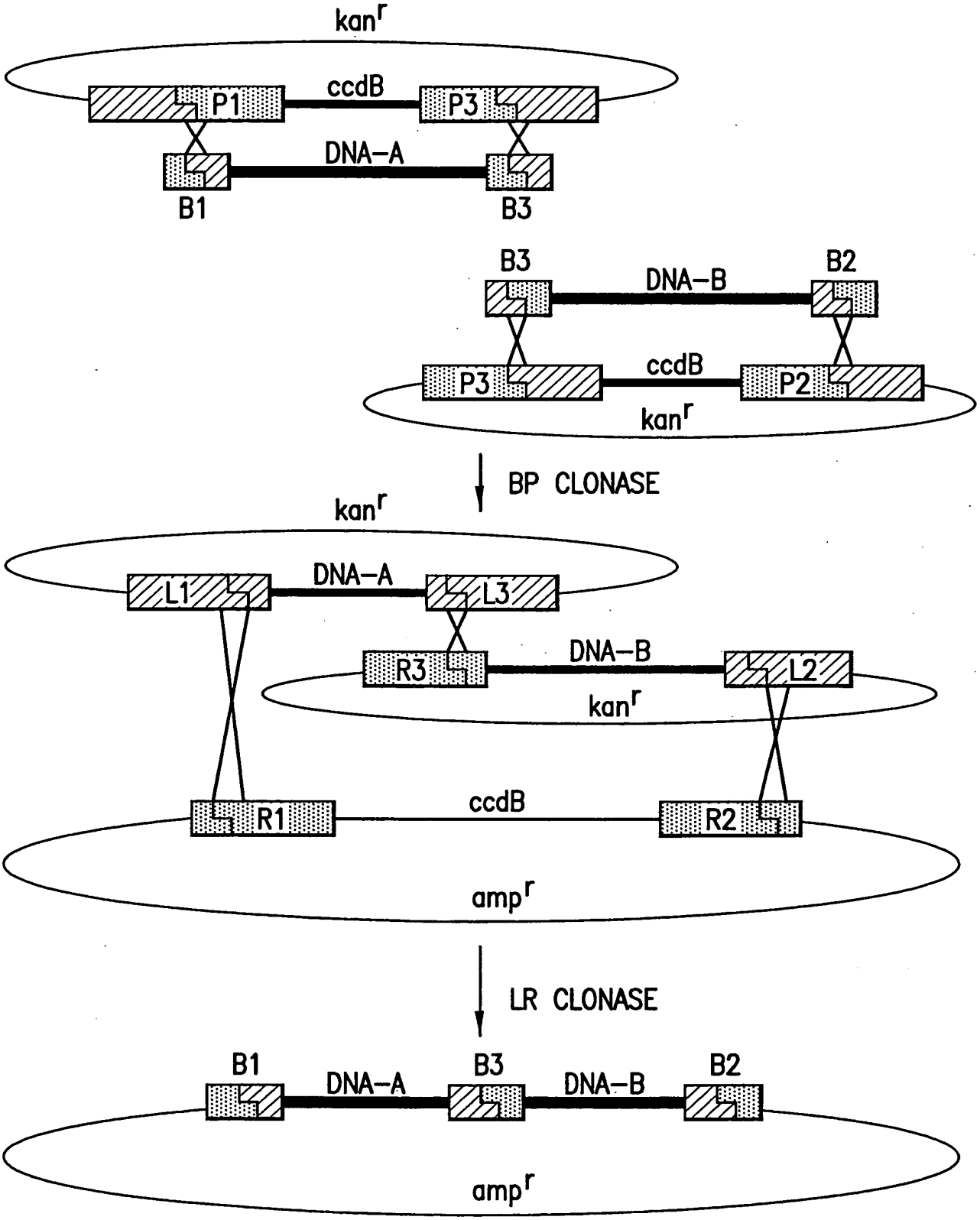

kan^r
P1
ccdB
P3
DNA–A
B1
B3
B3
DNA–B
B2
P3
ccdB
P2
kan^r
BP CLONASE
kan^r
L1
DNA–A
L3
R3
DNA–B
L2
kan^r
R1
ccdB
R2
amp^r
LR CLONASE
B1
DNA–A
B3
DNA–B
B2
amp^r

FIG. 4

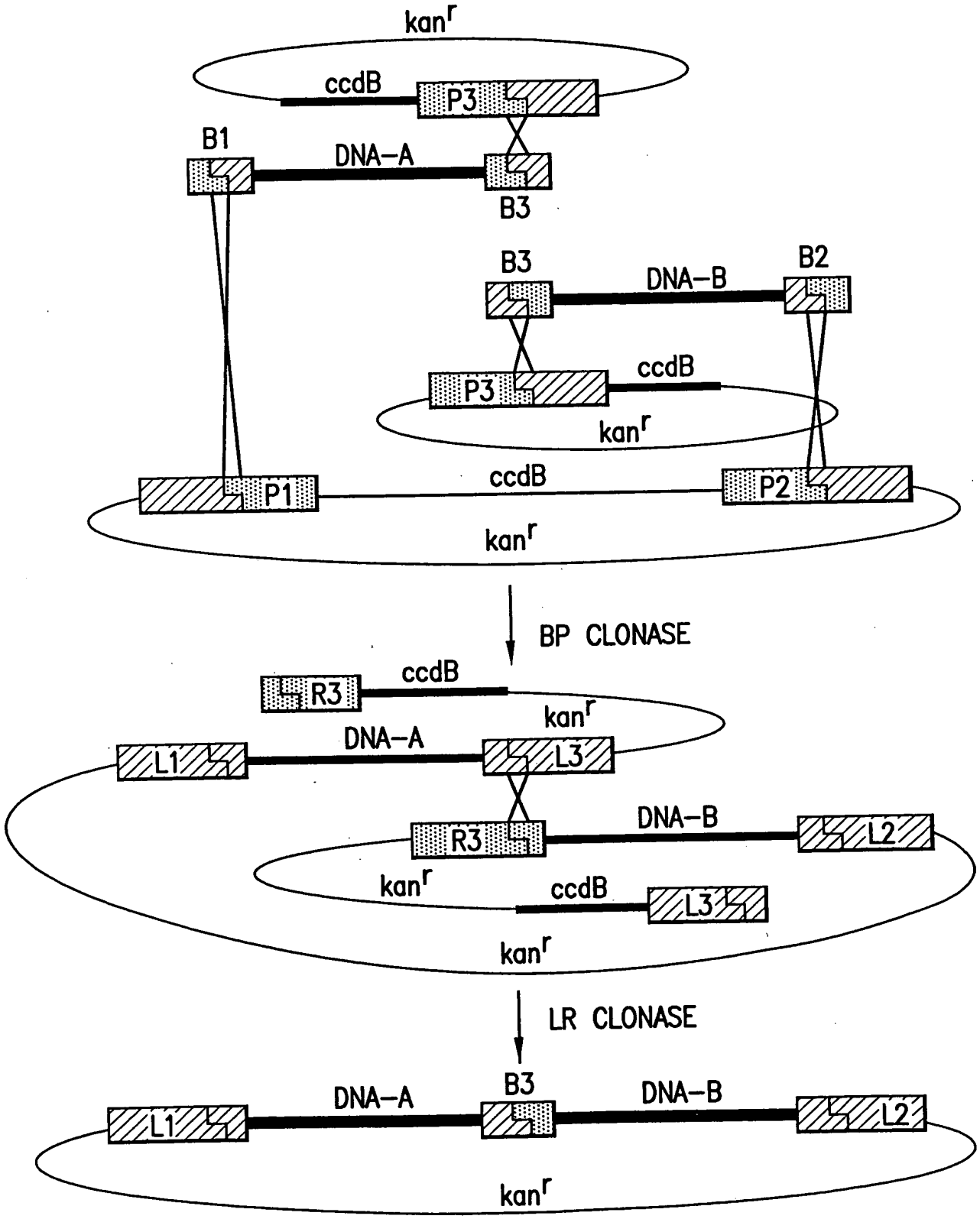

kan^r
ccdB
P3
B1
DNA–A
B3
B3
DNA–B
B2
ccdB
P3
kan^r
ccdB
P1
P2
kan^r
BP CLONASE
ccdB
R3
kan^r
DNA–A
L1
L3
DNA–B
R3
L2
kan^r
ccdB
L3
kan^r
LR CLONASE
B3
DNA–A
DNA–B
L1
L2
kan^r

FIG. 5

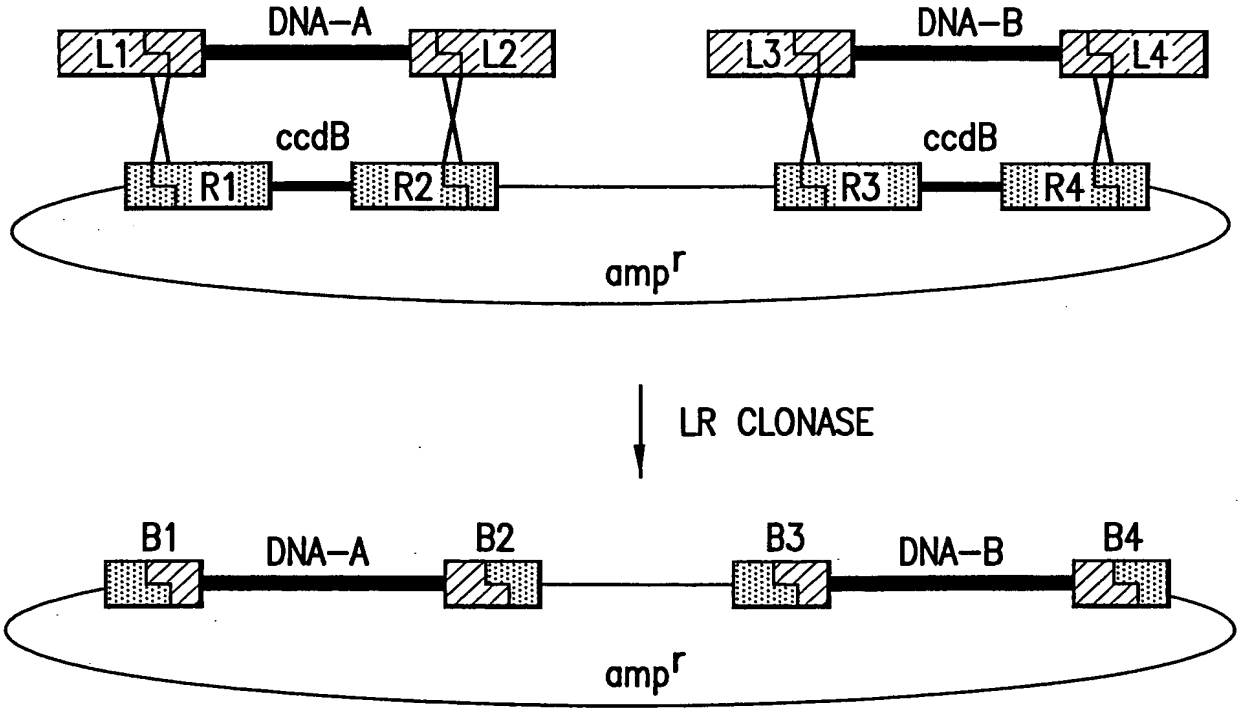

DNA–A
DNA–B
L1
L2
L3
L4
ccdB
ccdB
R1
R2
R3
R4
amp^r
LR CLONASE
B1
DNA–A
B2
B3
DNA–B
B4
amp^r

FIG. 6

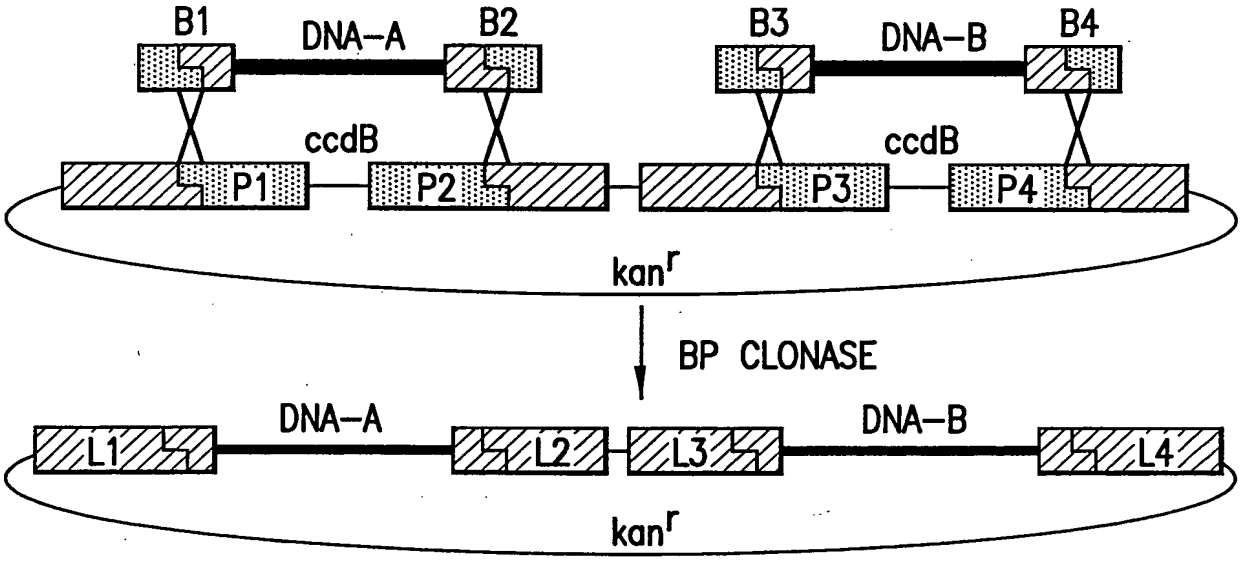

B1
DNA–A
B2
B3
DNA–B
B4
ccdB
ccdB
P1
P2
P3
P4
kan^r
BP CLONASE
DNA–A
DNA–B
L1
L2
L3
L4
kan^r

FIG. 7

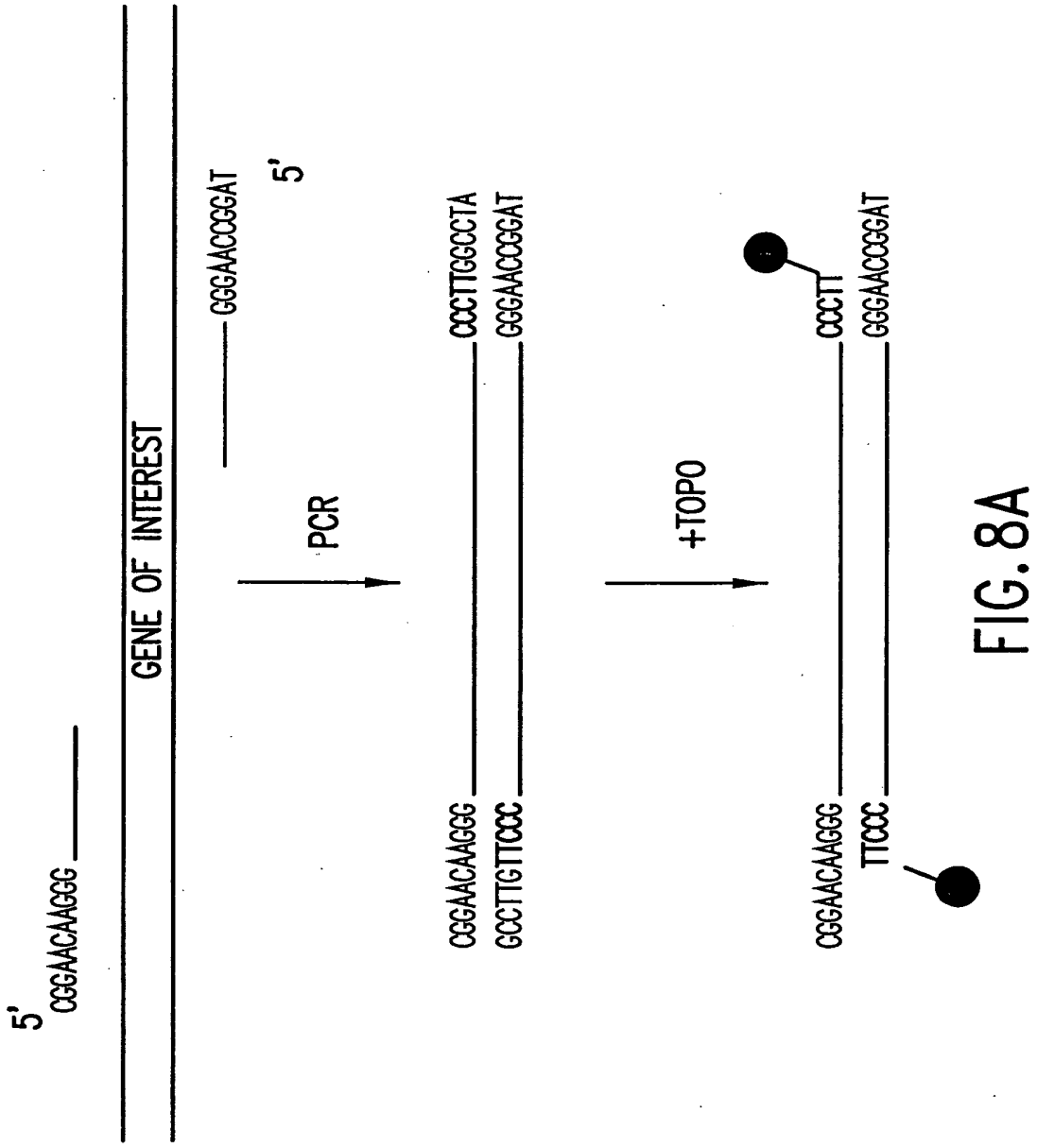

5'
CGGAACAAGGG
GENE OF INTEREST
GGGAACCGGAT
5'
PCR
CGGAACAAGGG
GCCTTGTTCCCC
CCCTTGGCCTA
GGGAACCGGAT
+TOPO
CGGAACAAGGG
TTCCCC
CCCTT
GGGAACCGGAT

FIG. 8A

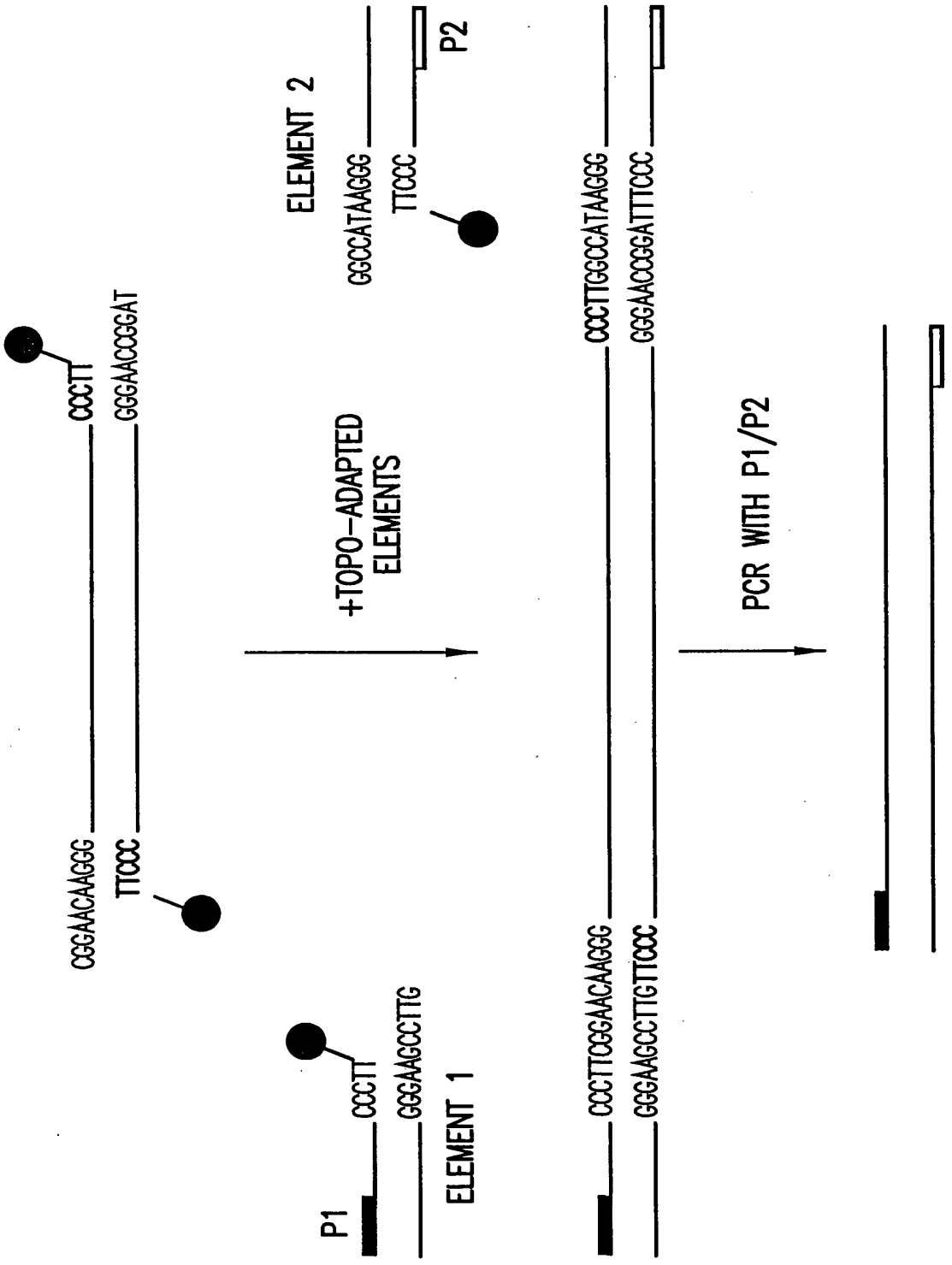

CCCTT
GGGAACCGGAT
CGGAACAAGGG
TTCCC
ELEMENT 2
GGCCATAAGGG
TTCCC
P2
+TOPO-ADAPTED ELEMENTS
P1
CCCTT
GGGAAGCCTTG
ELEMENT 1
CCCTTGGCCATAAGGG
GGGAACCGGATTTCCC
CCCTTCGGAACAAGGG
GGGAAGCCTTGTTCCC
PCR WITH P1/P2

FIG. 8B

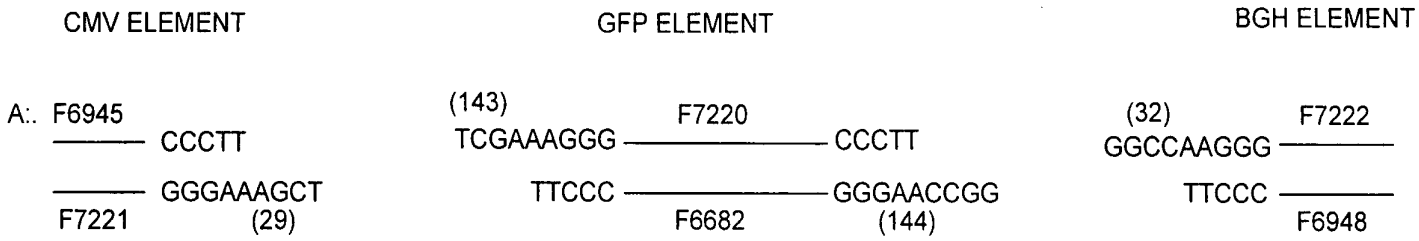


FIG. 9A

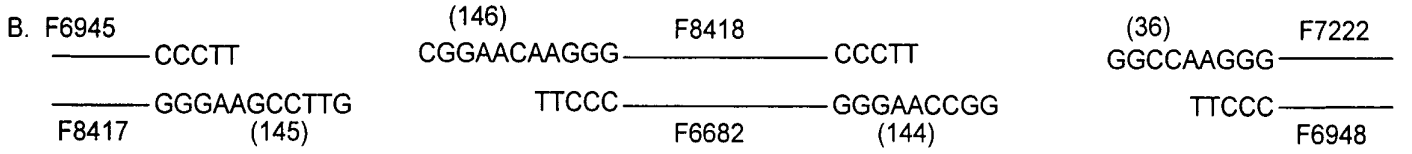


FIG. 9B

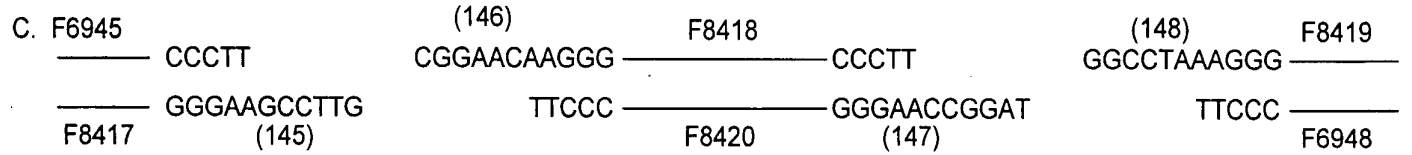


FIG. 9C

Sheet 10 of 59
Appl. No. 10/792,035; Filed: March 4, 2004
Dkt No. 0942.534005/BJD; Group Unit: 1645
Inventors: CHESNUT *et al.*; Tel: 202-371-2600
For: Methods and Compositions for Synthesis of Nucleic Acid Molecules Using Multiple Recognition Sites

TABLE 1

Primer name	F#	Sequence (5'-> 3')	SEQ ID NO:
MTH1	10779	TATGTATCATACACATACGATTTAGGT	1
MTH2	10780	ACCGCCTCTCCCCGCGCGTT	2
GAL4r2	12667	GTTCCGAAGGGGGCGATACAGTCAACTGTCTTTG	3
MTH5	12505	TTGGCCAAGGGTATCTAGAAGCTTCTGCAGACGCGT	4
VP16r2	12668	GTTCCGAAGGGCCACCGTACTCGTCAATTCCAAG	5
SV40pAf	12016	GGCCAAAAGGGAACTTGTTTATTGCAGCTTATAATG	6
SV40pAr	561	CTCTGACTTGAGCGTCGATTTT	7
p53f2	12669	CGGAACAAGGGGAATTCCCTGTCACCGAGACC	8
SVTf2	12670	CGGAACAAGGGGAATTCCCGGGGATCTGGAATTC	9
CMVr2	7221	TCGAAAGGGTCGAGGTCGACCTGCAGCTG	10
CMVf	6945	AATTCACATTGATTATTGAGTAGTTA	11
GFP-Xhof	7220	TCGAAAGGGTAATGGCCAGCAAAGGAGAAG	12
GFP-Notr	6682	GGCCAAGGGTTTGTAGAGCTCATCCAT	13
BGHf2	7222	GGCCAAGGGTCTGAATGGGGCCGCATAGT	14
BGHr	6948	AAGCCATAGAGCCCGGGCCA	15
CMVr3	8417	GTTCCGAAGGGTCGAGGTCGACCTGCAGCTG	16
GFPf3	8418	CGGAACAAGGGATGGCCAGCAAAGGAGAAG	17
GFPr3	8420	TAGGCCAAGGGTTTGTAGAGCTCATCCATGC	18
BGHf3	8419	GGCCTAAAGGGTGAATGGGGCCGCATAGT	19
T7top	9304	GAAGGAGTAATACGACTCACTATAGGGAGCCACCATGGGCCCTTCGGAAC	20
T7bottom	9305	GTTCCGAAGGGCCCATGGTGGCTCCCTATAGTGAGTCGTATTACTCCTTC	21
T7amp	9306	GAAGGAGTAATACGACTCACT	22
T3top	9661	GGCCTAAAGGGTCCCTTTAGTGAGGGTTAATTGCGCGC	23
T3bottom	9662	GCGCGCAATTAACCCTCACTAAAGGGACCCTTTAGGCC	24
lacZf2	10632	CGGAACAAGGGATGATAGATCCCGTCGTTTTACA	25
lacZ1k2	10770	TAGGCCAAGGGGACCATTTTCAATCCGCACCT	26
lacZ2k2	10771	TAGGCCAAGGGGAGGCACTTCACCGCTTGCCA	27
lacZ3k2	10772	TAGGCCAAGGGTTTGACACCAGACCAACTGGTA	28

FIG. 9D

SAMPLE #	GAL4+pA	VP16+pA	pGene/lacZ	GAL4+p53+pA	VP16+T+pA	p53-VP16
1			0.26 μg	p0.37 μg	p0.37 μg	
2			0.4 μg	p0.3 μg	p0.3 μg	
3			0.4 μg			p0.6 μg
4			0.4 μg	10.3 μg	10.3 μg	
5		10.3 μg	0.4 μg	10.3 μg		
6	10.3 μg		0.4 μg		10.3 μg	
7			0.4 μg	4.5 μl PCR	4.5 μl PCR	
8		4.5 μl PCR	0.4 μg	4.5 μl PCR		
9	4.5 μl PCR		0.4 μg		4.5 μl PCR	

FIG. 10A

SAMPLE #	LacZ activity
1	240000
2	140000
3	1800000
4	1400000
5	54000
6	80000
7	320000
8	12000
9	42000

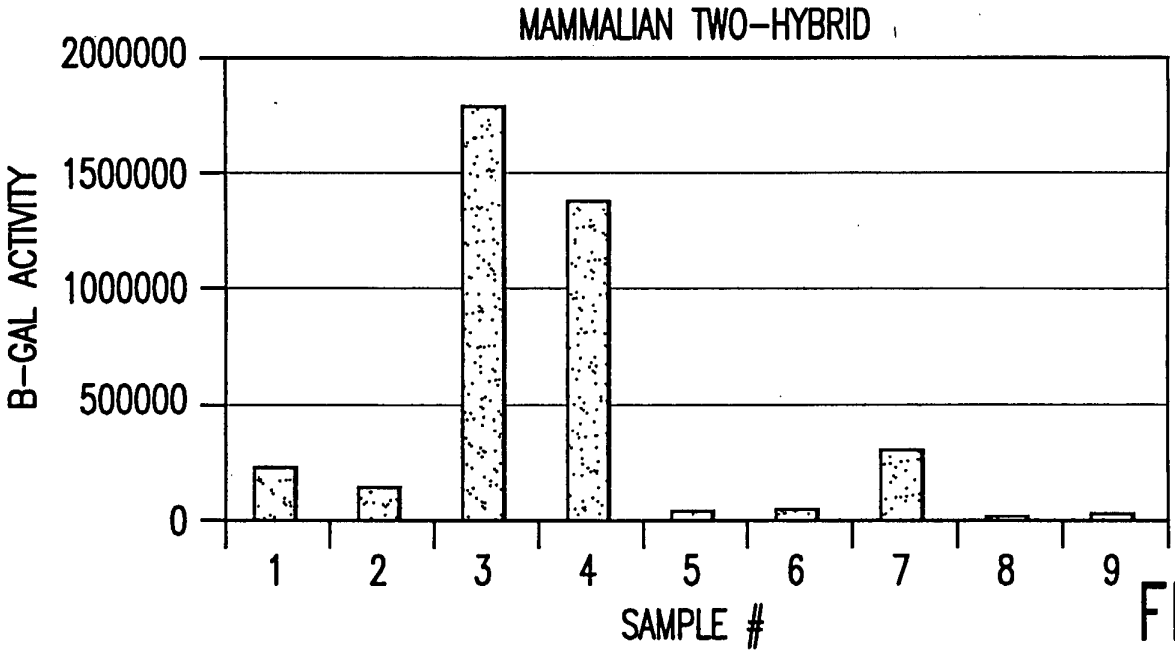


FIG. 10B

Sheet 12 of 59
Appl. No. 10/792,035; Filed: March 4, 2004
Dkt No. 0942.5340005/BJD; Group Unit: 1645
Inventors: CHESNUT *et al.*; Tel: 202-371-2600
For: Methods and Compositions for Synthesis of Nucleic Acid Molecules Using Multiple Recognition Sites

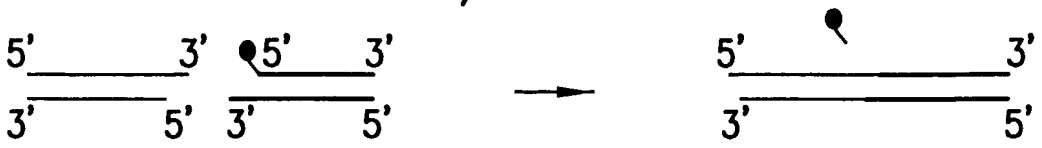


FIG. 11A

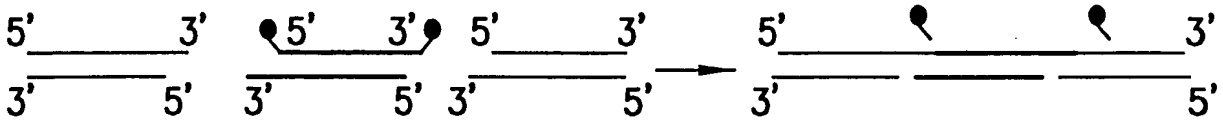


FIG. 11B

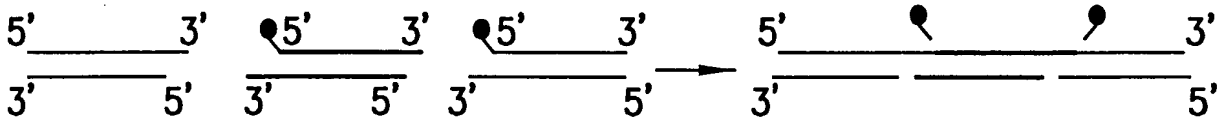


FIG. 11C

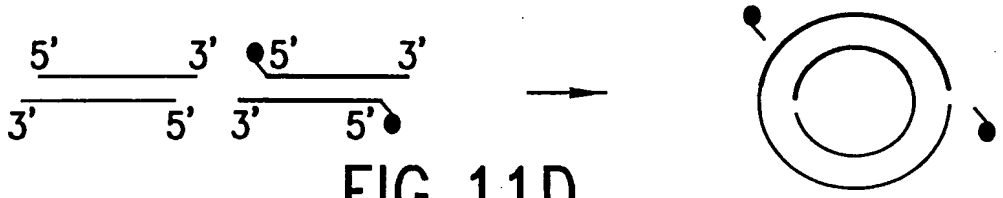


FIG. 11D

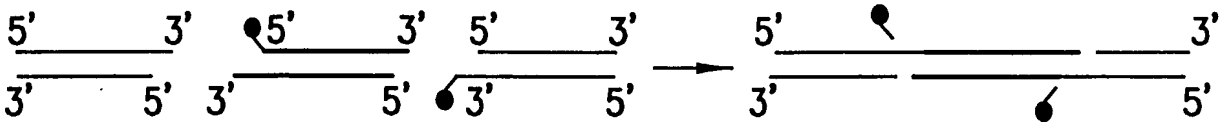


FIG. 11E

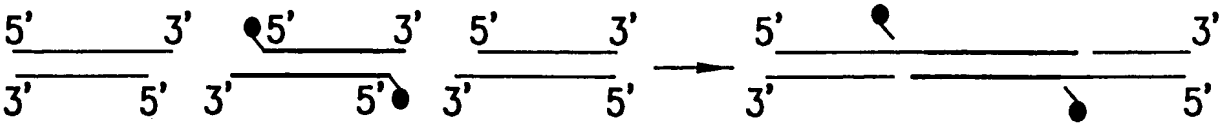


FIG. 11F

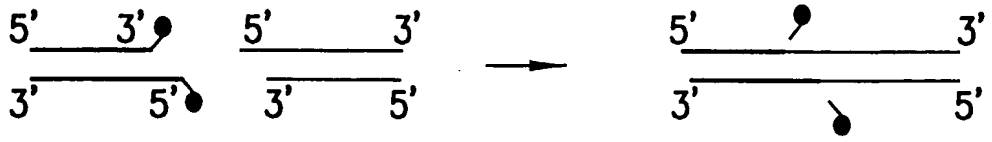


FIG. 12A

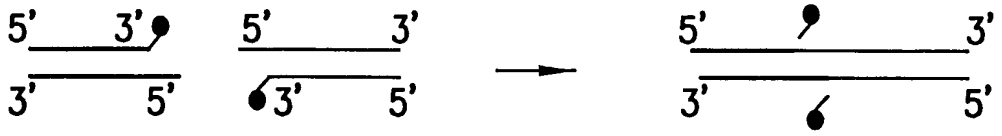


FIG. 12B

FIG. 12C

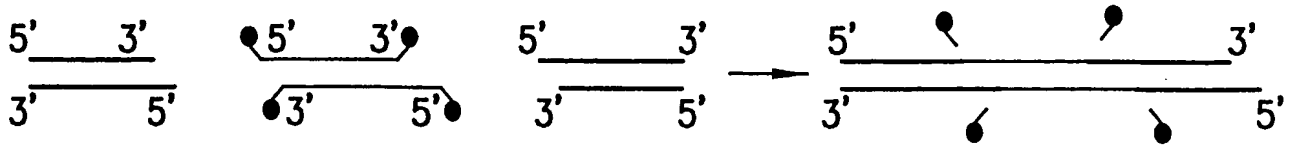


FIG. 12D

15/59

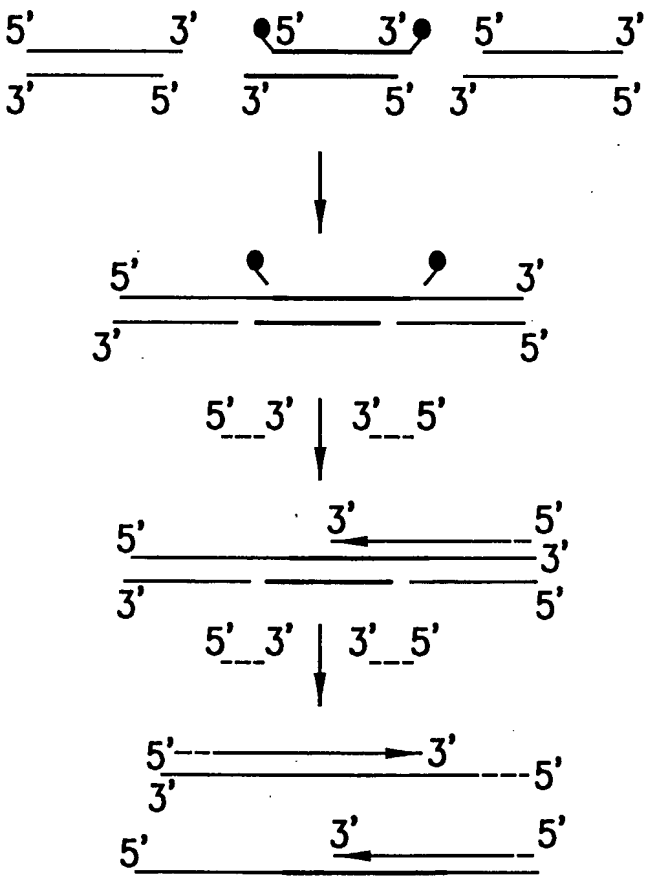


FIG. 13

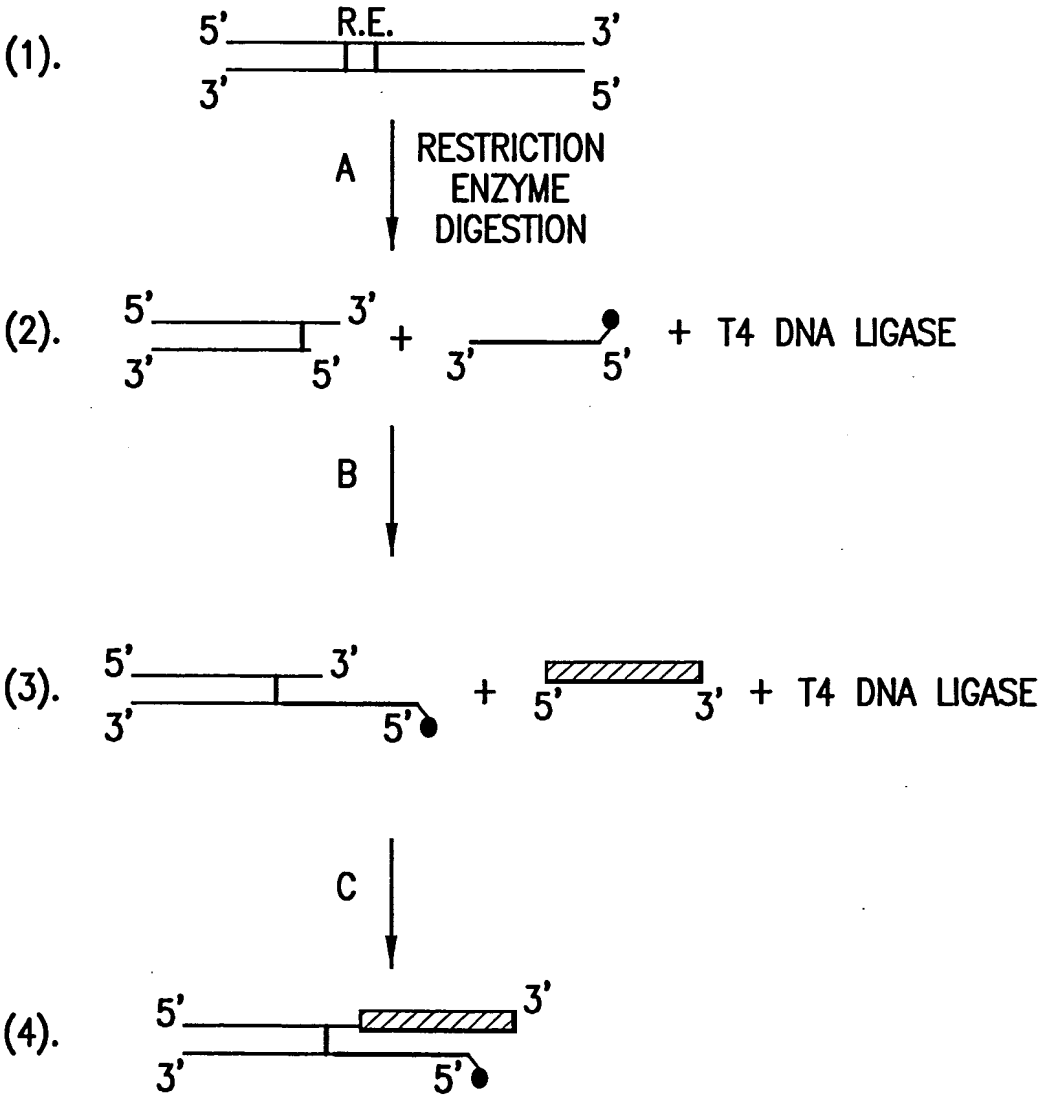

(1).
5'
R.E.
3'
3'
5'
A
RESTRICTION ENZYME DIGESTION
(2).
5'
3'
3'
5'
+
3'
5'
+ T4 DNA LIGASE
B
(3).
5'
3'
3'
5'
+
5'
3'
+ T4 DNA LIGASE
C
(4).
5'
3'
3'
5'

FIG. 14

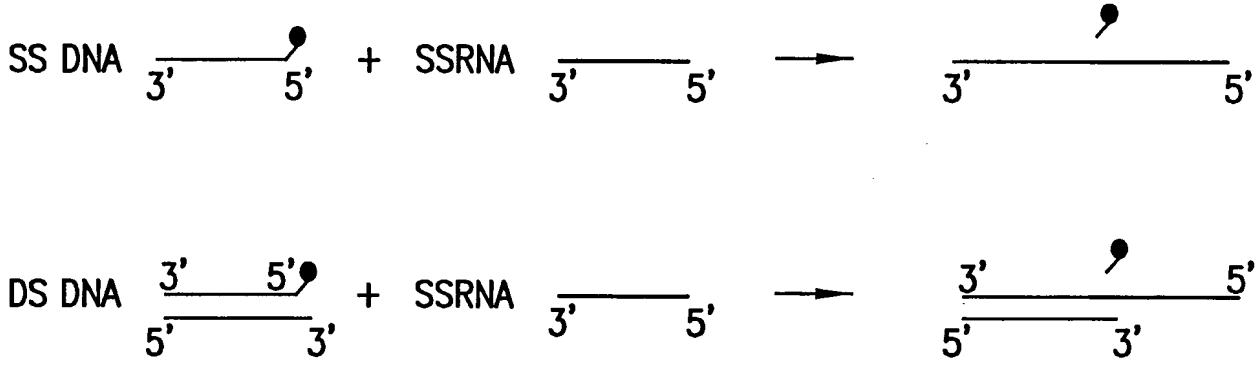


FIG. 15

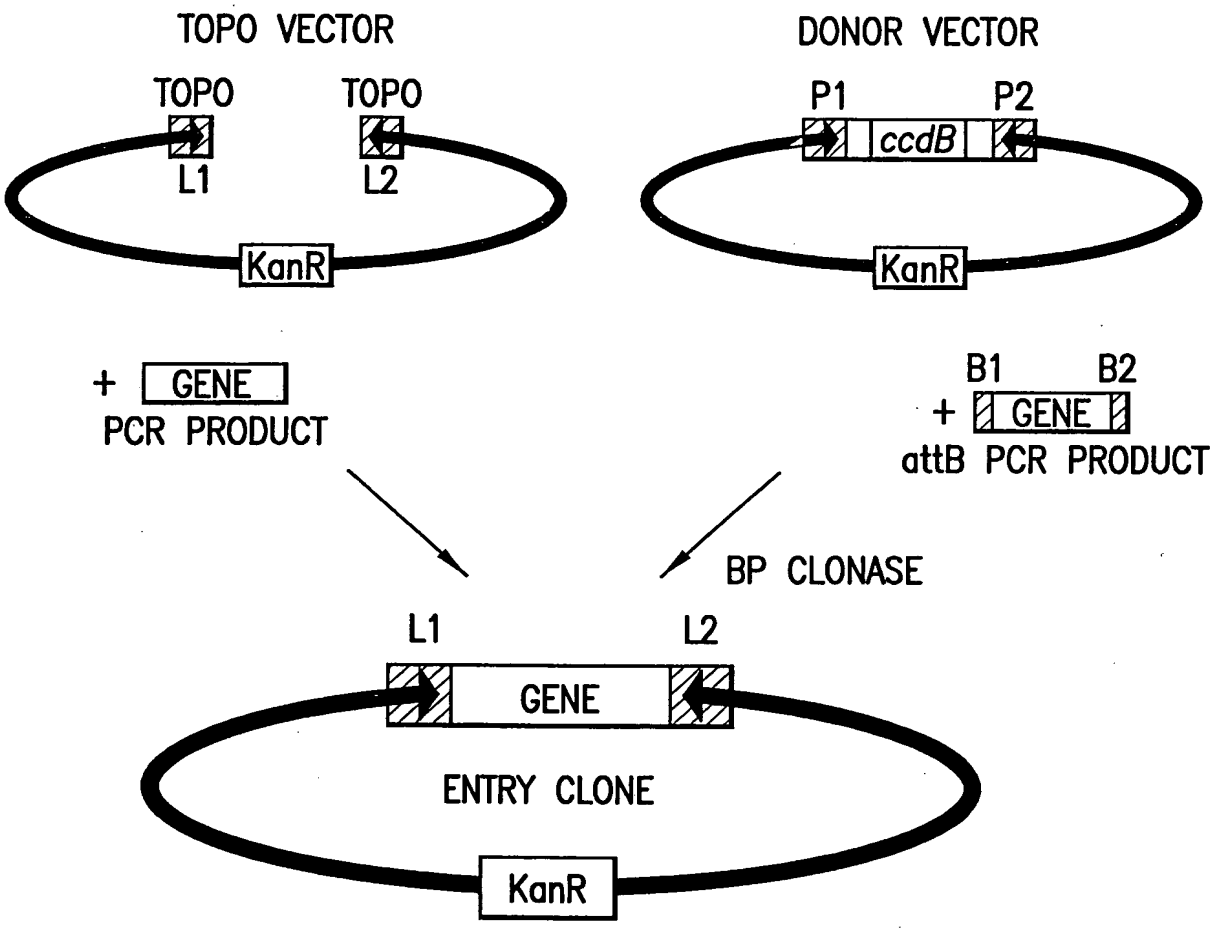

TOPO VECTOR
TOPO
TOPO
L1
L2
KanR
DONOR VECTOR
P1
P2
ccdB
KanR
+ GENE
PCR PRODUCT
B1
B2
+ GENE
attB PCR PRODUCT
BP CLONASE
L1
L2
GENE
ENTRY CLONE
KanR

FIG. 16

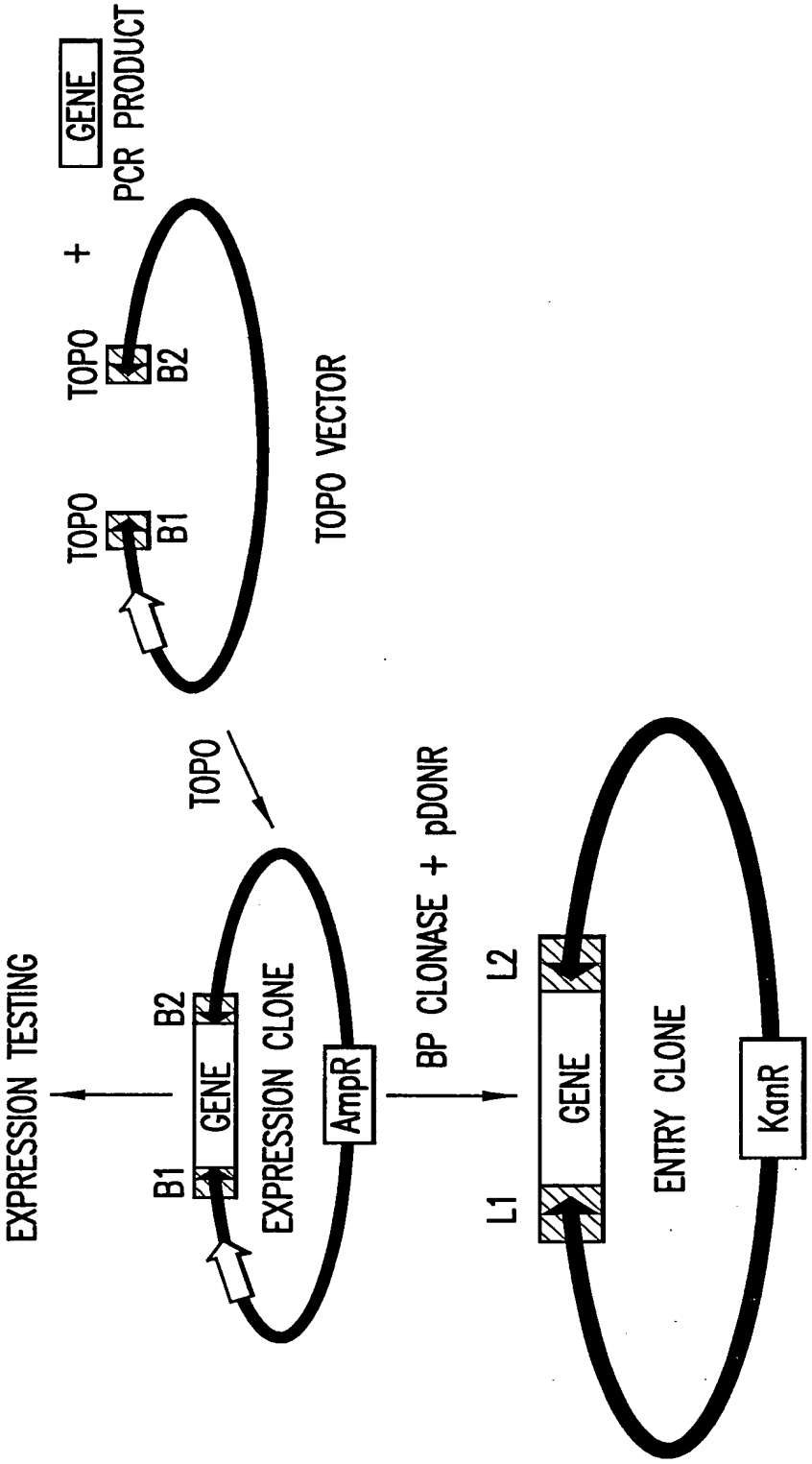

GENE
PCR PRODUCT
+
TOPO
B2
TOPO
B1
TOPO VECTOR
TOPO
BP CLONASE + pDONR
L2
L1
GENE
ENTRY CLONE
KanR
EXPRESSION TESTING
B2
B1
GENE
EXPRESSION CLONE
AmpR

FIG. 17

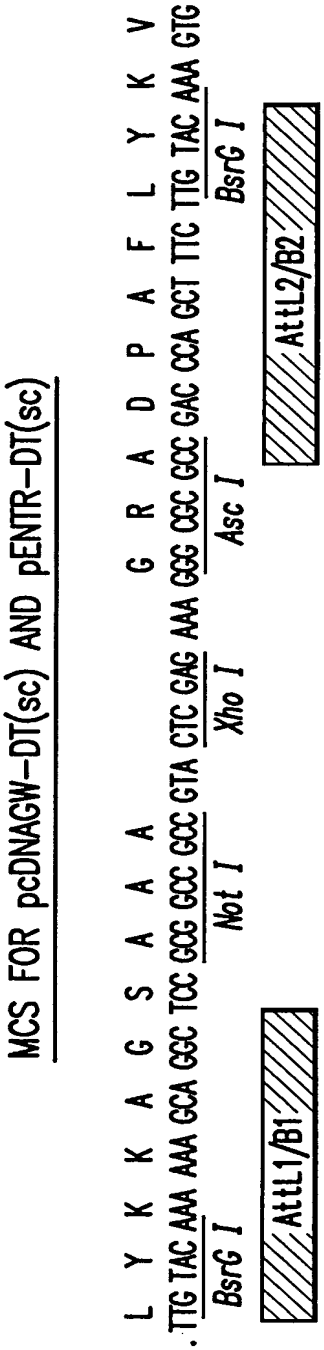

MCS FOR pcDNAGW-DT(sc) AND pENTR-DT(sc)
L Y K K A G S A A A G R A D P A F L Y K V
...TTG TAC AAA AAA GCA GGC TCC GCG GCC GCC GTA CTC GAG AAA GGG CGC GCC GAC CCA GCT TTC TTG TAC AAA GTG
BsrG I
Not I
Xho I
Asc I
BsrG I
AttL1/B1
AttL2/B2

FIG. 18

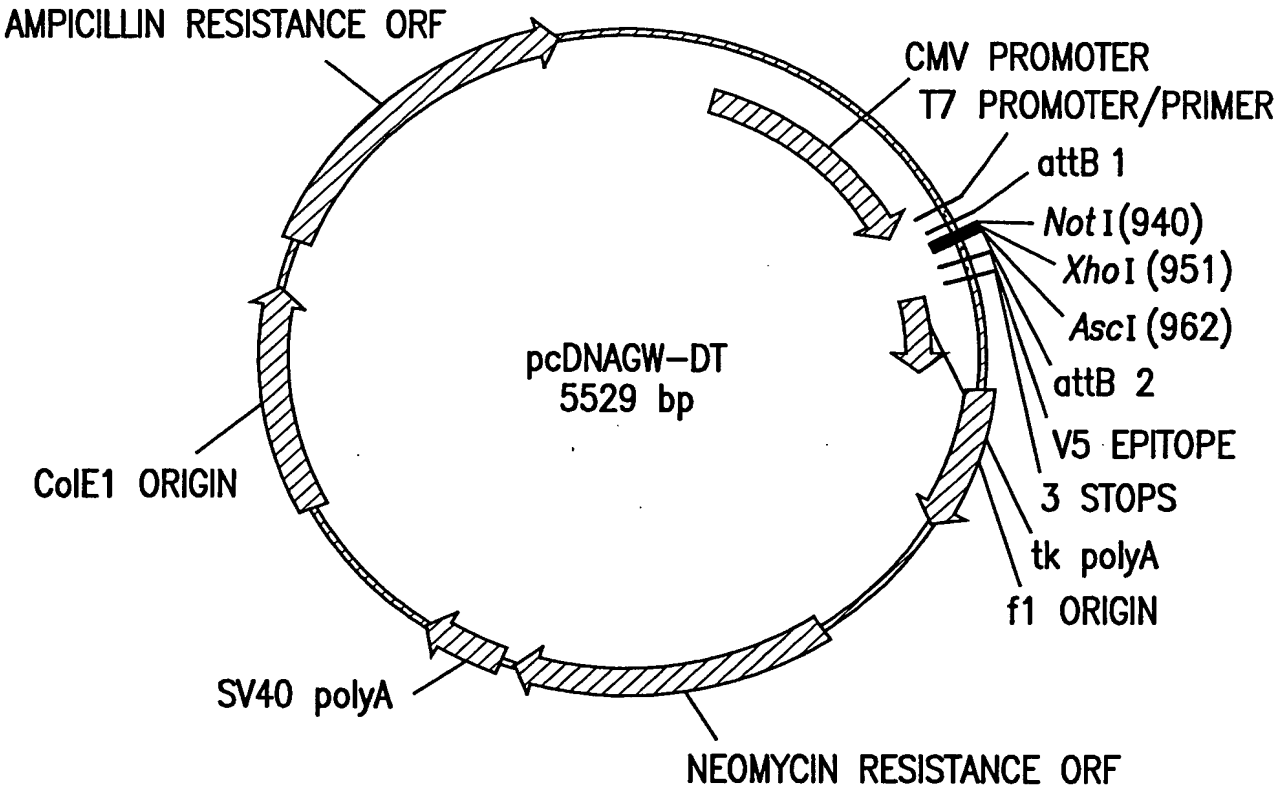

AMPICILLIN RESISTANCE ORF
CMV PROMOTER
T7 PROMOTER/PRIMER
attB 1
NotI(940)
XhoI (951)
AscI (962)
attB 2
V5 EPITOPE
3 STOPS
tk polyA
f1 ORIGIN
pcDNAGW-DT
5529 bp
ColE1 ORIGIN
SV40 polyA
NEOMYCIN RESISTANCE ORF

FIG. 19

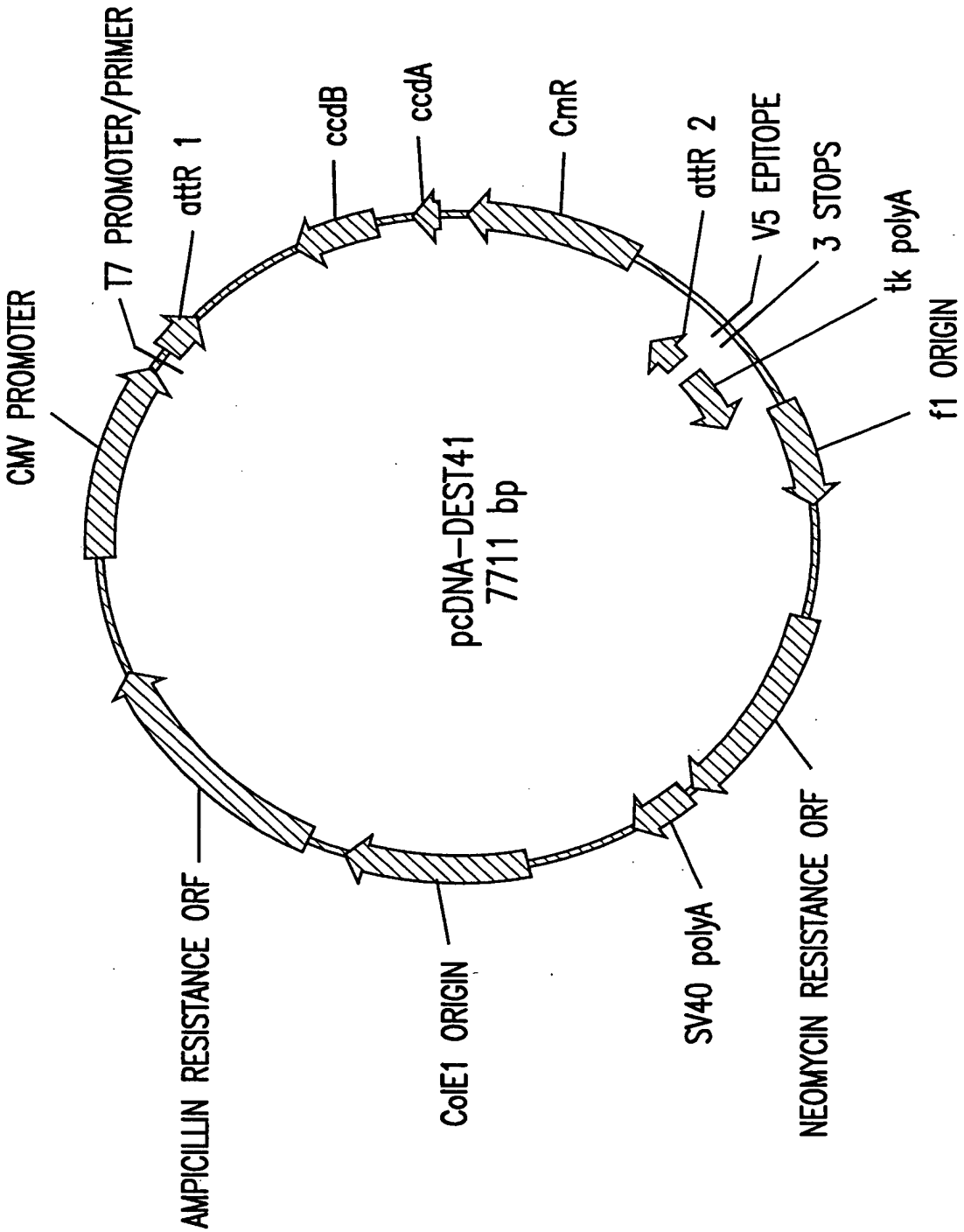

CMV PROMOTER
T7 PROMOTER/PRIMER
attR 1
ccdB
ccdA
CmR
attR 2
V5 EPITOPE
3 STOPS
tk polyA
f1 ORIGIN
pcDNA-DEST41
7711 bp
AMPICILLIN RESISTANCE ORF
ColE1 ORIGIN
SV40 polyA
NEOMYCIN RESISTANCE ORF

FIG. 20

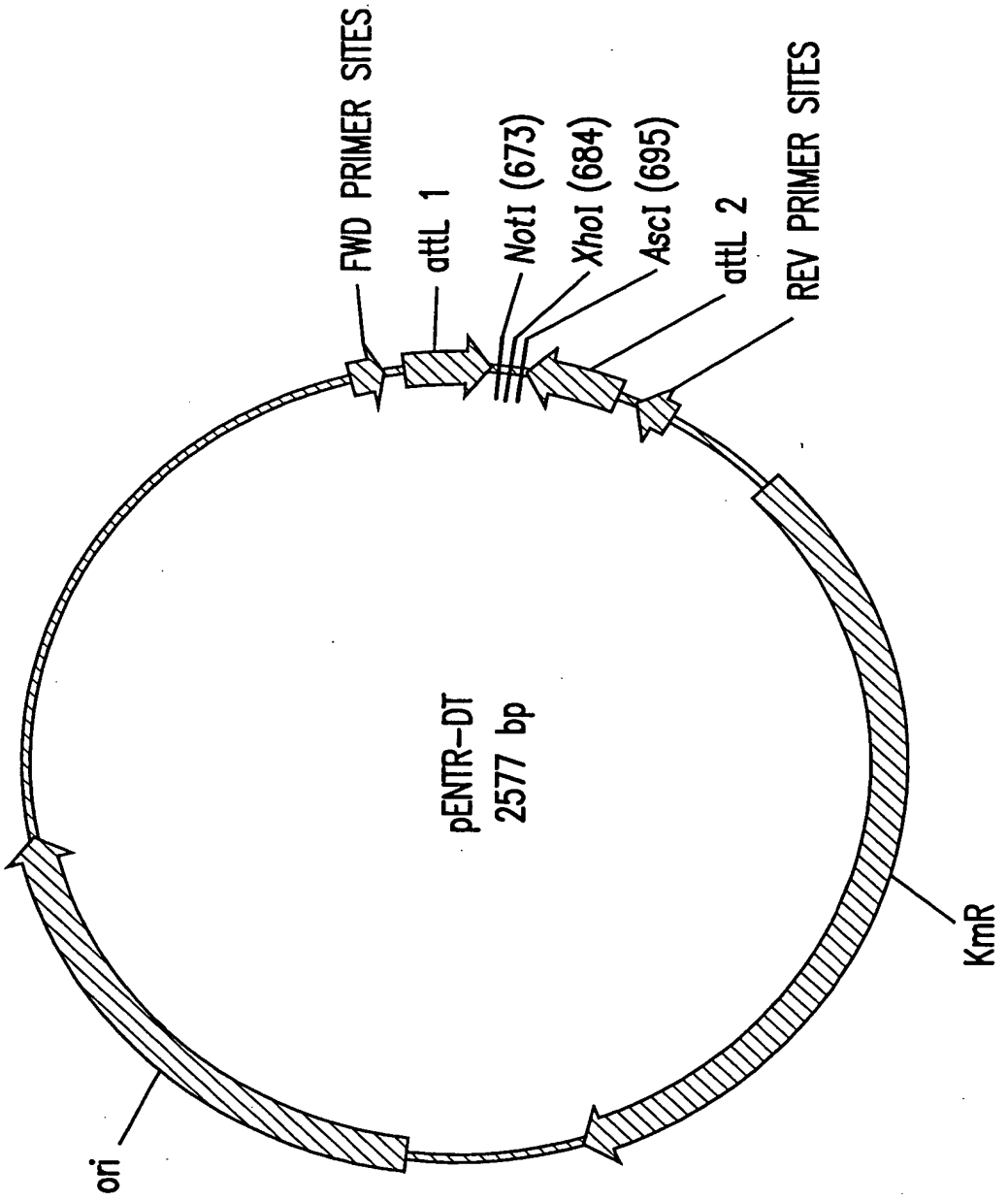

FWD PRIMER SITES
attL 1
NotI (673)
XhoI (684)
AscI (695)
attL 2
REV PRIMER SITES
pENTR-DT
2577 bp
KmR
ori

FIG. 21

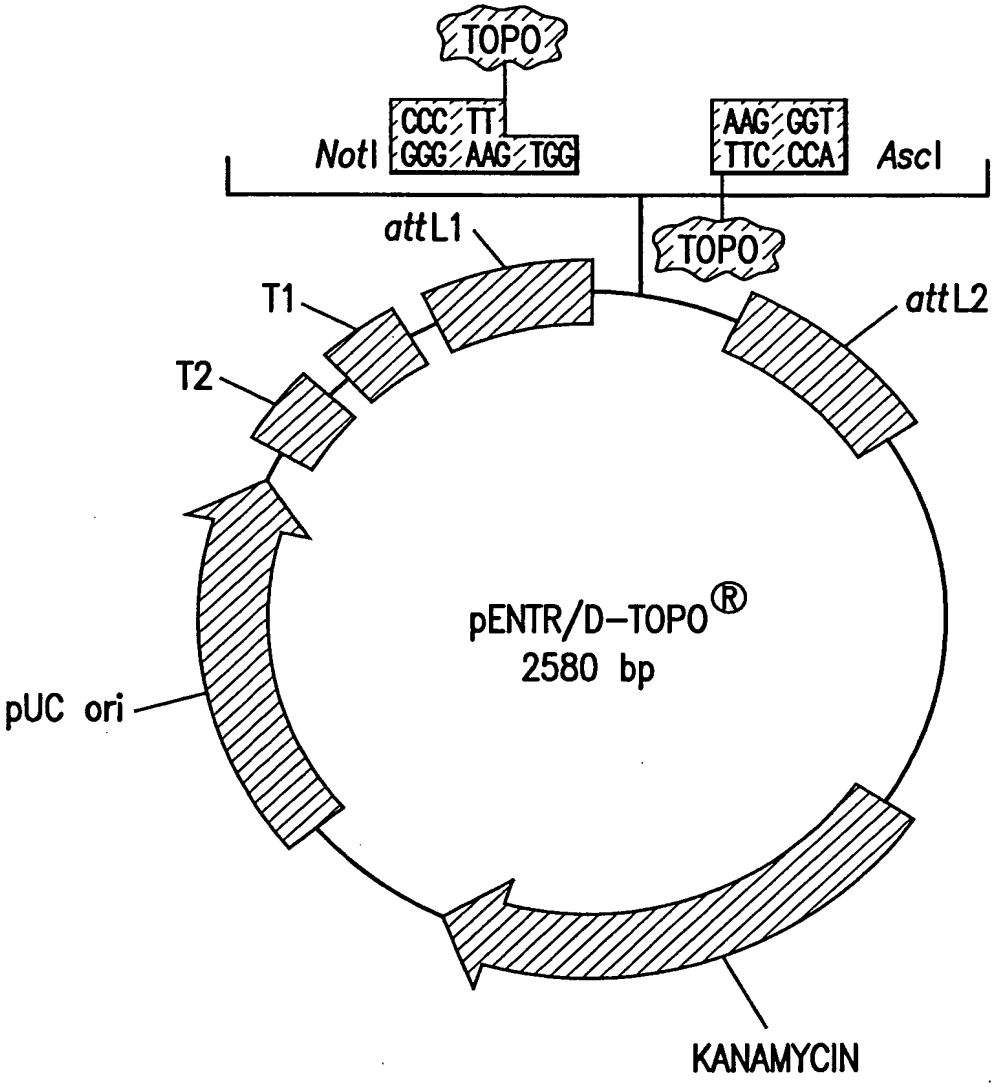

TOPO
CCC TT
GGG AAG TGG
NotI
AAG GGT
TTC CCA
AscI
attL1
TOPO
attL2
T1
T2
pENTR/D-TOPO®
2580 bp
pUC ori
KANAMYCIN

FIG. 22A

```
   1 ctttcctgcg ttatcccctg attctgtgga taaccgtatt accgcctttg agtgagctga
  61 taccgctcgc cgcagccgaa cgaccgagcg cagcgagtca gtgagcgagg aagcggaaga
 121 gcgcccaata cgcaaaccgc ctctccccgc gcgttggccg attcattaat gcagctggca
 181 cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaata cgcgtaccgc
 241 tagccaggaa gagtttgtag aaacgcaaaa aggccatccg tcaggatggc cttctgctta
 301 gtttgatgcc tggcagttta tggcgggcgt cctgcccgcc accctccggg ccgttgcttc
 361 acaacgttca aatccgctcc cggcggattt gtcctactca ggagagcgtt caccgacaaa
 421 caacagataa aacgaaaggc ccagtcttcc gactgagcct ttcgttttat ttgatgcctg
 481 gcagttccct actctcgcgt taacgctagc atggatgttt tcccagtcac gacgttgtaa
 541 aacgacggcc agtcttaagc tcgggcccca aataatgatt ttattttgac tgatagtgac
 601 ctgttcgttg caacaaattg atgagcaatg cttttttata atgccaactt tgtacaaaaa
 661 agcaggctcc gcggccgccc cttcaccatg nnnnnnnnna agggtgggcg cgccgaccca
 721 gctttcttgt acaaagttgg cattataaga aagcattgct tatcaatttg ttgcaacgaa
 781 caggtcacta tcagtcaaaa taaaatcatt atttgccatc cagctgatat cccctatagt
 841 gagtcgtatt acatggtcat agctgtttcc tggcagctct ggcccgtgtc tcaaaatctc
 901 tgatgttaca ttgcacaaga taaaaatata tcatcatgaa caataaaact gtctgcttac
 961 ataaacagta atacaagggg tgttatgagc catattcaac gggaaacgtc gaggccgcga
1021 ttaaattcca acatggatgc tgatttatat gggtataaat gggctcgcga taatgtcggg
1081 caatcaggtg cgacaatcta tcgcttgtat gggaagcccg atgcgccaga gttgtttctg
1141 aaacatggca aaggtagcgt tgccaatgat gttacagatg agatggtcag actaaactgg
1201 ctgacggaat ttatgcctct tccgaccatc aagcatttta tccgtactcc tgatgatgca
1261 tggttactca ccactgcgat ccccggaaaa acagcattcc aggtattaga agaatatcct
1321 gattcaggtg aaaatattgt tgatgcgctg gcagtgttcc tgcgccggtt gcattcgatt
1381 cctgtttgta attgtccttt taacagcgat cgcgtatttc gtctcgctca ggcgcaatca
1441 cgaatgaata acggtttggt tgatgcgagt gattttgatg acgagcgtaa tggctggcct
1501 gttgaacaag tctggaaaga aatgcataaa cttttgccat tctcaccgga ttcagtcgtc
1561 actcatggtg atttctcact tgataacctt atttttgacg aggggaaatt aataggttgt
1621 attgatgttg gacgagtcgg aatcgcagac cgataccagg atcttgccat cctatggaac
1681 tgcctcggtg agttttctcc ttcattacag aaacggcttt ttcaaaaata tggtattgat
1741 aatcctgata tgaataaatt gcagtttcat ttgatgctcg atgagttttt ctaatcagaa
1801 ttggttaatt ggttgtaaca ctggcagagc attacgctga cttgacggga cggcgcaagc
1861 tcatgaccaa aatcccttaa cgtgagttac gcgtcgttcc actgagcgtc agaccccgta
1921 gaaaagatca aaggatcttc ttgagatcct ttttttctgc gcgtaatctg ctgcttgcaa
1981 acaaaaaaac caccgctacc agcggtggtt tgtttgccgg atcaagagct accaactctt
2041 tttccgaagg taactggctt cagcagagcg cagataccaa atactgtcct tctagtgtag
2101 ccgtagttag gccaccactt caagaactct gtagcaccgc ctacatacct cgctctgcta
2161 atcctgttac cagtggctgc tgccagtggc gataagtcgt gtcttaccgg gttggactca
2221 agacgatagt taccggataa ggcgcagcgg tcgggctgaa cgggggttc gtgcacacag
2281 cccagcttgg agcgaacgac ctacaccgaa ctgagatacc tacagcgtga gcattgagaa
2341 agcgccacgc ttcccgaagg gagaaaggcg gacaggtatc cggtaagcgg cagggtcgga
2401 acaggagagc gcacgaggga gcttccaggg ggaaacgcct ggtatcttta tagtcctgtc
2461 gggtttcgcc acctctgact tgagcgtcga tttttgtgat gctcgtcagg ggggcggagc
2521 ctatggaaaa acgccagcaa cgcggccttt ttacggttcc tggccttttg ctggccttt
2581 gctcacatgt t
```

FIG.22B

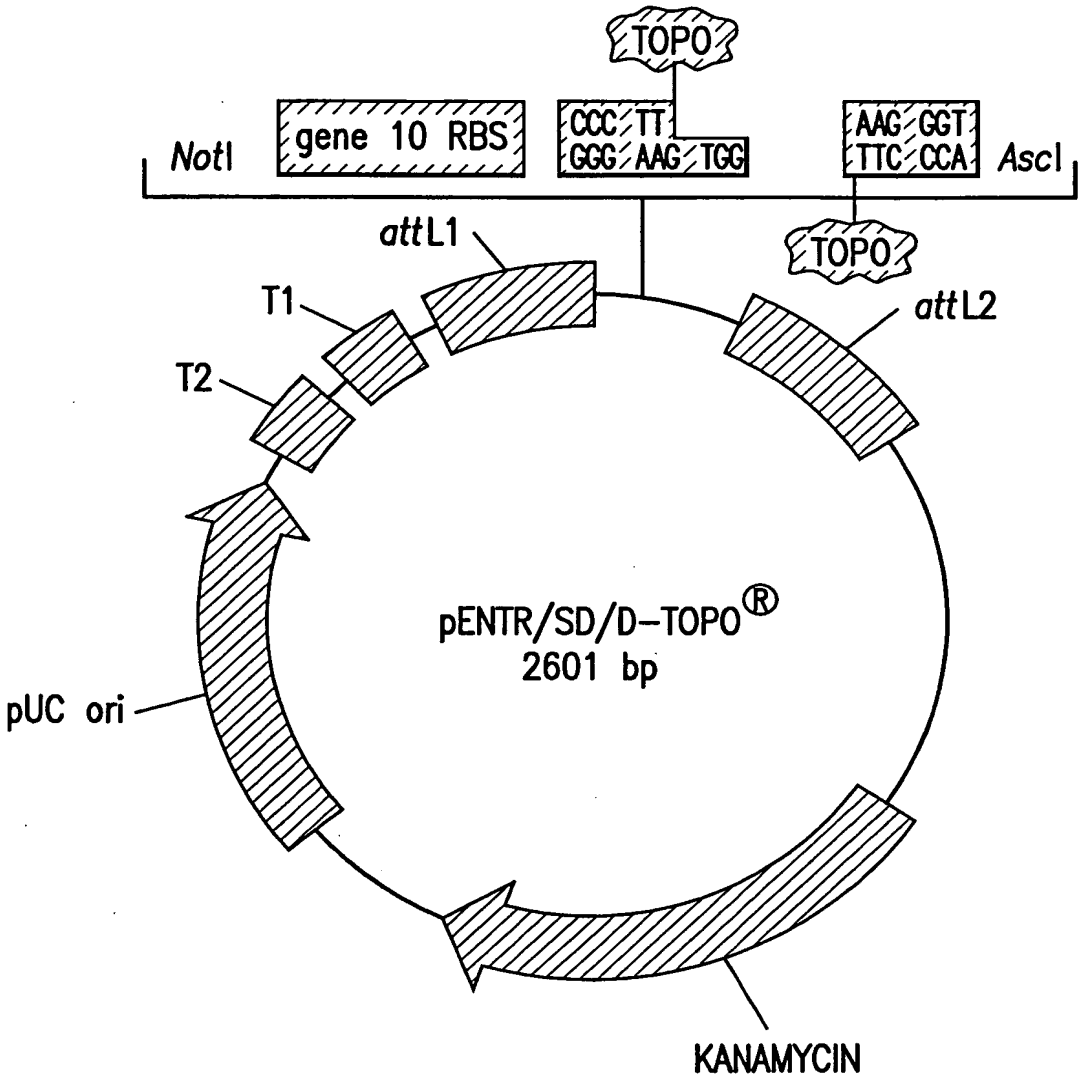

TOPO
NotI
gene 10 RBS
CCC TT
GGG AAG TGG
AAG GGT
TTC CCA
AscI
attL1
TOPO
T1
attL2
T2
pENTR/SD/D-TOPO®
2601 bp
pUC ori
KANAMYCIN

FIG. 23A

```
   1 ctttcctgcg ttatcccctg attctgtgga taaccgtatt accgcctttg agtgagctga
  61 taccgctcgc cgcagccgaa cgaccgagcg cagcgagtca gtgagcgagg aagcggaaga
 121 gcgcccaata cgcaaaccgc ctctccccgc gcgttggccg attcattaat gcagctggca
 181 cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaata cgcgtaccgc
 241 tagccaggaa gagtttgtag aaacgcaaaa aggccatccg tcaggatggc cttctgctta
 301 gtttgatgcc tggcagttta tggcgggcgt cctgcccgcc accctccggg ccgttgcttc
 361 acaacgttca aatccgctcc cggcggattt gtcctactca ggagagcgtt caccgacaaa
 421 caacagataa aacgaaaggc ccagtcttcc gactgagcct ttcgttttat ttgatgcctg
 481 gcagttccct actctcgcgt taacgctagc atggatgttt tcccagtcac gacgttgtaa
 541 aacgacggcc agtcttaagc tcgggcccca aataatgatt ttattttgac tgatagtgac
 601 ctgttcgttg caacaaattg atgagcaatg ctttttttata atgccaactt tgtacaaaaa
 661 agcaggctcc gcggccgcct tgtttaactt taagaaggag cccttcaccn nnnnnaaggg
 721 tgggcgcgcc gacccagctt tcttgtacaa agttggcatt ataagaaagc attgcttatc
 781 aatttgttgc aacgaacagg tcactatcag tcaaaataaa atcattattt gccatccagc
 841 tgatatcccc tatagtgagt cgtattacat ggtcatagct gtttcctggc agctctggcc
 901 cgtgtctcaa aatctctgat gttacattgc acaagataaa aatatatcat catgaacaat
 961 aaaactgtct gcttacataa acagtaatac aaggggtgtt atgagccata ttcaacggga
1021 aacgtcgagg ccgcgattaa attccaacat ggatgctgat ttatatgggt ataaatgggc
1081 tcgcgataat gtcgggcaat caggtgcgac aatctatcgc ttgtatggga agcccgatgc
1141 gccagagttg tttctgaaac atggcaaagg tagcgttgcc aatgatgtta cagatgagat
1201 ggtcagacta aactggctga cggaatttat gcctcttccg accatcaagc attttatccg
1261 tactcctgat gatgcatggt tactcaccac tgcgatcccc ggaaaaacag cattccaggt
1321 attagaagaa tatcctgatt caggtgaaaa tattgttgat gcgctggcag tgttcctgcg
1381 ccggttgcat tcgattcctg tttgtaattg tccttttaac agcgatcgcg tatttcgtct
1441 cgctcaggcg caatcacgaa tgaataacgg tttggttgat gcgagtgatt ttgatgacga
1501 gcgtaatggc tggcctgttg aacaagtctg gaaagaaatg cataaacttt tgccattctc
1561 accggattca gtcgtcactc atggtgattt ctcacttgat aaccttattt ttgacgaggg
1621 gaaattaata ggttgtattg atgttggacg agtcggaatc gcagaccgat accaggatct
1681 tgccatccta tggaactgcc tcggtgagtt ttctccttca ttacagaaac ggctttttca
1741 aaaatatggt attgataatc ctgatatgaa taaattgcag tttcatttga tgctcgatga
1801 gtttttctaa tcagaattgg ttaattggtt gtaacactgg cagagcatta cgctgacttg
1861 acgggacggc gcaagctcat gaccaaaatc ccttaacgtg agttacgcgt cgttccactg
1921 agcgtcagac cccgtagaaa agatcaaagg atcttcttga gatccttttt ttctgcgcgt
1981 aatctgctgc ttgcaaacaa aaaaaccacc gctaccagcg gtggtttgtt tgccggatca
2041 agagctacca actcttttc cgaaggtaac tggcttcagc agagcgcaga taccaaatac
2101 tgtccttcta gtgtagccgt agttaggcca ccacttcaag aactctgtag caccgcctac
2161 atacctcgct ctgctaatcc tgttaccagt ggctgctgcc agtggcgata agtcgtgtct
2221 taccgggttg gactcaagac gatagttacc ggataaggcg cagcggtcgg gctgaacggg
2281 gggttcgtgc acacagccca gcttggagcg aacgacctac accgaactga gatacctaca
2341 gcgtgagcat tgagaaagcg ccacgcttcc cgaagggaga aaggcggaca ggtatccggt
2401 aagcggcagg gtcggaacag gagagcgcac gagggagctt ccagggggaa acgcctggta
2461 tctttatagt cctgtcgggt ttcgccacct ctgacttgag cgtcgatttt tgtgatgctc
2521 gtcaggggg cggagcctat ggaaaaacgc cagcaacgcg gccttttac ggttcctggc
2581 cttttgctgg ccttttgctc acatgtt
```

FIG.23B

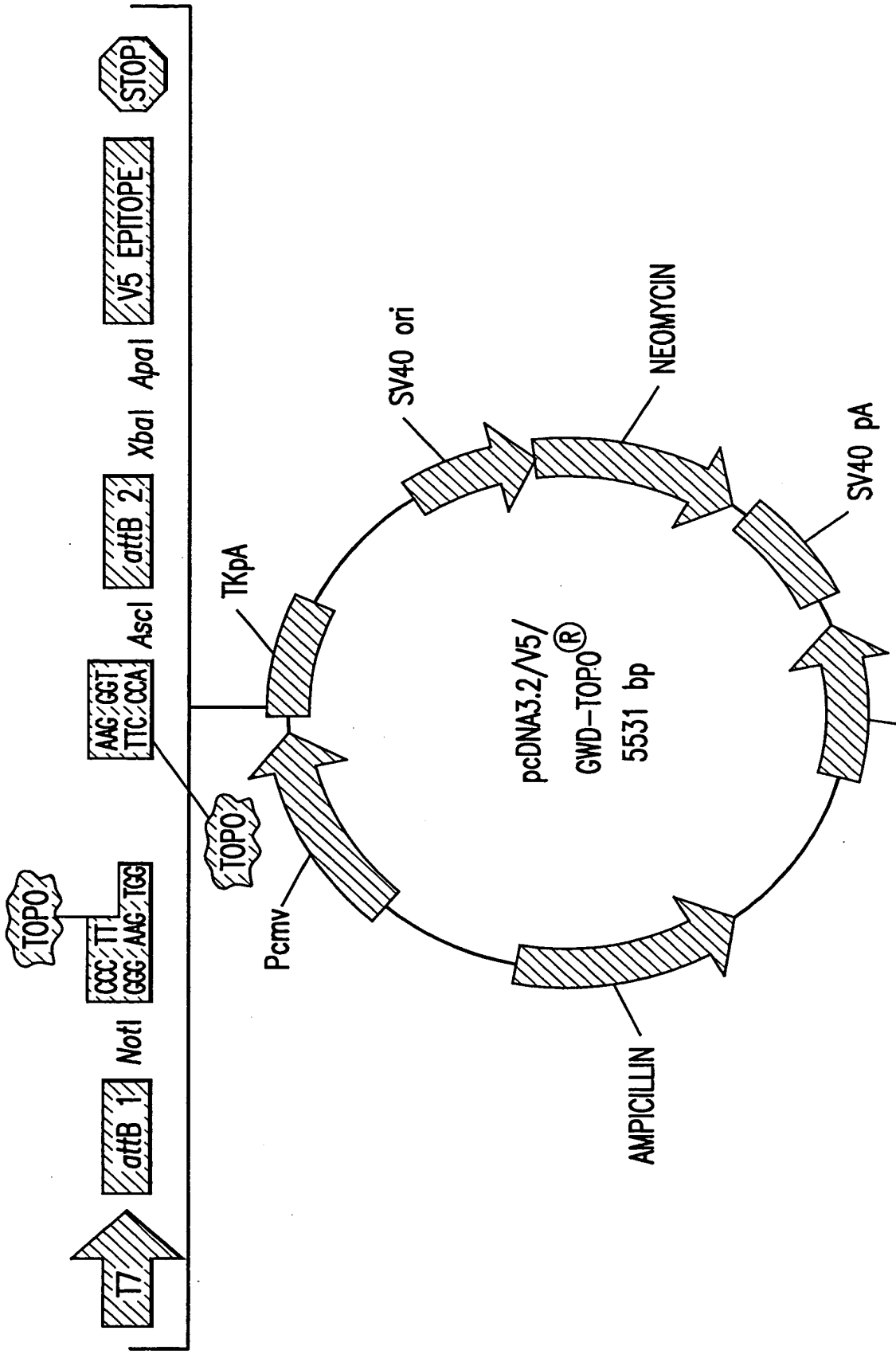

T7
attB 1
NotI
CCC TT
GGG AAG TGG
TOPO
TOPO
AAG GGT
TTC CCA
AscI
attB 2
XbaI
ApaI
V5 EPITOPE
STOP
TKpA
Pcmv
SV40 ori
NEOMYCIN
SV40 pA
pUC ori
AMPICILLIN
pcDNA3.2/V5/
GWD-TOPO®
5531 bp

FIG. 24A

```
   1 gacggatcgg gagatctccc gatcccctat ggtcgactct cagtacaatc tgctctgatg
  61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
 121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
 181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
 241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
 301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
 361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
 421 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt
 481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
 541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca
 601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
 661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
 721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
 781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
 841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagt
 901 taagctatca acaagtttgt acaaaaaagc aggctccgcg gccgcccctt caccatgnnn
 961 nnnnnnaagg gtgggcgcgc cgacccagct ttcttgtaca aagtggttga tctagagggc
1021 ccgcggttcg aaggtaagcc tatccctaac cctctcctcg gtctcgattc tacgcgtacc
1081 ggttagtaat gagtttaaac gggggaggct aactgaaaca cggaaggaga caataccgga
1141 aggaacccgc gctatgacgg caataaaaag acagaataaa acgcacgggt gttgggtcgt
1201 ttgttcataa acgcggggtt cggtcccagg gctggcactc tgtcgatacc ccaccgagac
1261 cccattgggg ccaatacgcc cgcgtttctt ccttttcccc accccacccc ccaagttcgg
1321 gtgaaggccc agggctcgca gccaacgtcg gggcggcagg ccctgccata gcagatctgc
1381 gcagctgggg ctctaggggg tatccccacg cgccctgtag cggcgcatta agcgcggcgg
1441 gtgtggtggt tacgcgcagc gtgaccgcta cacttgccag cgccctagcg cccgctcctt
1501 tcgctttctt cccttccttt ctcgccacgt tcgccggctt tccccgtcaa gctctaaatc
1561 ggggcatccc tttagggttc cgatttagtg ctttacggca cctcgacccc aaaaaacttg
1621 attagggtga tggttcacgt agtgggccat cgccctgata gacggttttt cgccctttga
1681 cgttggagtc cacgttcttt aatagtggac tcttgttcca aactggaaca acactcaacc
1741 ctatctcggt ctattctttt gatttataag ggattttggg gatttcggcc tattggttaa
1801 aaaatgagct gatttaacaa aaatttaacg cgaattaatt ctgtggaatg tgtgtcagtt
1861 agggtgtgga aagtccccag gctccccagc aggcagaagt atgcaaagca tgcatctcaa
1921 ttagtcagca accaggtgtg gaaagtcccc aggctcccca gcaggcagaa gtatgcaaag
1981 catgcatctc aattagtcag caaccatagt cccgccccta actccgccca tcccgccccc
2041 aactccgccc agttccgccc attctccgcc ccatggctga ctaatttttt ttatttatgc
2101 agaggccgag gccgcctctg cctctgagct attccagaag tagtgaggag gcttttttgg
2161 aggcctaggc ttttgcaaaa agctcccggg agcttgtata tccattttcg gatctgatca
2221 agagacagga tgaggatcgt ttcgcatgat tgaacaagat ggattgcacg caggttctcc
2281 ggccgcttgg gtggagaggc tattcggcta tgactgggca caacagacaa tcggctgctc
2341 tgatgccgcc gtgttccggc tgtcagcgca ggggcgcccg gttctttttg tcaagaccga
2401 cctgtccggt gccctgaatg aactgcagga cgaggcagcg cggctatcgt ggctggccac
2461 gacgggcgtt ccttgcgcag ctgtgctcga cgttgtcact gaagcgggaa gggactggct
2521 gctattgggc gaagtgccgg ggcaggatct cctgtcatct caccttgctc ctgccgagaa
2581 agtatccatc atggctgatg caatgcggcg gctgcatacg cttgatccgg ctacctgccc
2641 attcgaccac caagcgaaac atcgcatcga gcgagcacgt actcggatgg aagccggtct
2701 tgtcgatcag gatgatctgg acgaagagca tcaggggctc gcgccagccg aactgttcgc
2761 caggctcaag gcgcgcatgc ccgacggcga ggatctcgtc gtgacccatg gcgatgcctg-
```

FIG. 24B

2821 cttgccgaat atcatggtgg aaaatggccg cttttctgga ttcatcgact gtggccggct
2881 gggtgtggcg gaccgctatc aggacatagc gttggctacc cgtgatattg ctgaagagct
2941 tggcggcgaa tgggctgacc gcttcctcgt gctttacggt atcgccgctc ccgattcgca
3001 gcgcatcgcc ttctatcgcc ttcttgacga gttcttctga gcgggactct ggggttcgcg
3061 aaatgaccga ccaagcgacg cccaacctgc catcacgaga tttcgattcc accgccgcct
3121 tctatgaaag gttgggcttc ggaatcgttt tccgggacgc cggctggatg atcctccagc
3181 gcggggatct catgctggag ttcttcgccc accccaactt gtttattgca gcttataatg
3241 gttacaaata aagcaatagc atcacaaatt tcacaaataa agcatttttt tcactgcatt
3301 ctagttgtgg tttgtccaaa ctcatcaatg tatcttatca tgtctgtata ccgtcgacct
3361 ctagctagag cttggcgtaa tcatggtcat agctgtttcc tgtgtgaaat tgttatccgc
3421 tcacaattcc acacaacata cgagccggaa gcataaagtg taaagcctgg ggtgcctaat
3481 gagtgagcta actcacatta attgcgttgc gctcactgcc cgctttccag tcgggaaacc
3541 tgtcgtgcca gctgcattaa tgaatcggcc aacgcgcggg gagaggcggt ttgcgtattg
3601 ggcgctcttc cgcttcctcg ctcactgact cgctgcgctc ggtcgttcgg ctgcggcgag
3661 cggtatcagc tcactcaaag gcggtaatac ggttatccac agaatcaggg gataacgcag
3721 gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa ccgtaaaaag gccgcgttgc
3781 tggcgttttt ccataggctc cgccccctg acgagcatca caaaaatcga cgctcaagtc
3841 agaggtggcg aaacccgaca ggactataaa gataccaggc gtttccccct ggaagctccc
3901 tcgtgcgctc tcctgttccg accctgccgc ttaccggata cctgtccgcc tttctccctt
3961 cgggaagcgt ggcgctttct caatgctcac gctgtaggta tctcagttcg gtgtaggtcg
4021 ttcgctccaa gctgggctgt gtgcacgaac cccccgttca gcccgaccgc tgcgccttat
4081 ccggtaacta tcgtcttgag tccaacccgg taagacacga cttatcgcca ctggcagcag
4141 ccactggtaa caggattagc agagcgaggt atgtaggcgg tgctacagag ttcttgaagt
4201 ggtggcctaa ctacggctac actagaagga cagtatttgg tatctgcgct ctgctgaagc
4261 cagttacctt cggaaaaaga gttggtagct cttgatccgg caaacaaacc accgctggta
4321 gcggtggttt ttttgtttgc aagcagcaga ttacgcgcag aaaaaaagga tctcaagaag
4381 atcctttgat cttttctacg gggtctgacg ctcagtggaa cgaaaactca cgttaaggga
4441 ttttggtcat gagattatca aaaaggatct tcacctagat ccttttaaat taaaaatgaa
4501 gttttaaatc aatctaaagt atatatgagt aaacttggtc tgacagttac caatgcttaa
4561 tcagtgaggc acctatctca gcgatctgtc tatttcgttc atccatagtt gcctgactcc
4621 ccgtcgtgta gataactacg atacgggagg gcttaccatc tggccccagt gctgcaatga
4681 taccgcgaga cccacgctca ccggctccag atttatcagc aataaaccag ccagccggaa
4741 gggccgagcg cagaagtggt cctgcaactt tatccgcctc catccagtct attaattgtt
4801 gccgggaagc tagagtaagt agttcgccag ttaatagttt gcgcaacgtt gttgccattg
4861 ctacaggcat cgtggtgtca cgctcgtcgt ttggtatggc ttcattcagc tccggttccc
4921 aacgatcaag gcgagttaca tgatccccca tgttgtgcaa aaaagcggtt agctccttcg
4981 gtcctccgat cgttgtcaga agtaagttgg ccgcagtgtt atcactcatg gttatggcag
5041 cactgcataa ttctcttact gtcatgccat ccgtaagatg cttttctgtg actggtgagt
5101 actcaaccaa gtcattctga gaatagtgta tgcggcgacc gagttgctct tgcccggcgt
5161 caatacggga taataccgcg ccacatagca gaactttaaa agtgctcatc attggaaaac
5221 gttcttcggg gcgaaaactc tcaaggatct taccgctgtt gagatccagt tcgatgtaac
5281 ccactcgtgc acccaactga tcttcagcat cttttacttt caccagcgtt tctgggtgag
5341 caaaaacagg aaggcaaaat gccgcaaaaa agggaataag ggcgacacgg aaatgttgaa
5401 tactcatact cttccttttt caatattatt gaagcattta tcagggttat tgtctcatga
5461 gcggatacat atttgaatgt atttagaaaa ataaacaaat aggggttccg cgcacatttc
5521 cccgaaaagt gccacctgac gtc

FIG.24C

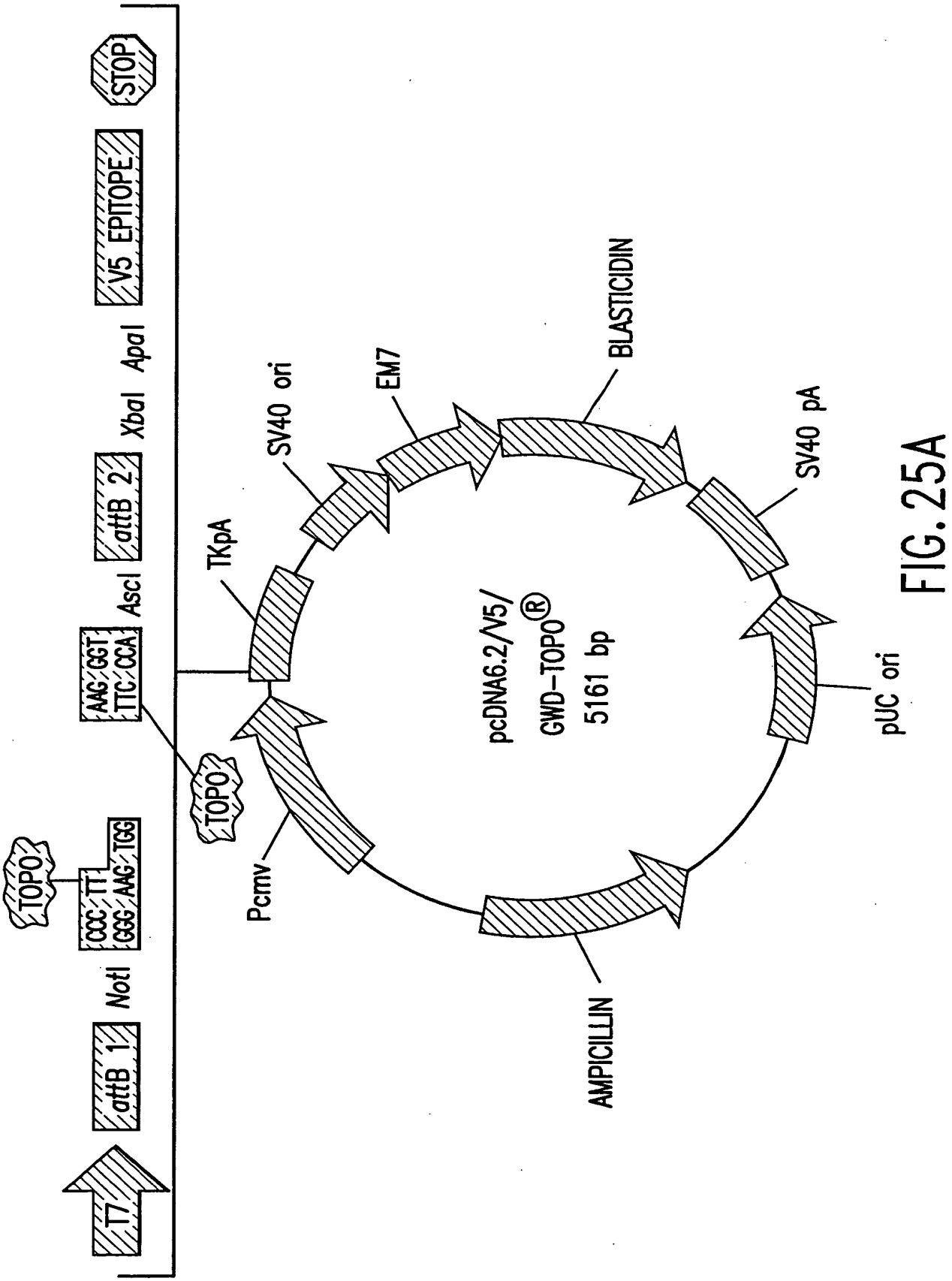

T7
attB 1
NotI
CCC TT
GGG AAG TGG
TOPO
TOPO
AAG GGT
TTC CCA
AscI
attB 2
XbaI
ApaI
V5 EPITOPE
STOP
TKpA
SV40 ori
EM7
BLASTICIDIN
SV40 pA
pUC ori
AMPICILLIN
Pcmv
pcDNA6.2/V5/
GWD-TOPO®
5161 bp

FIG. 25A

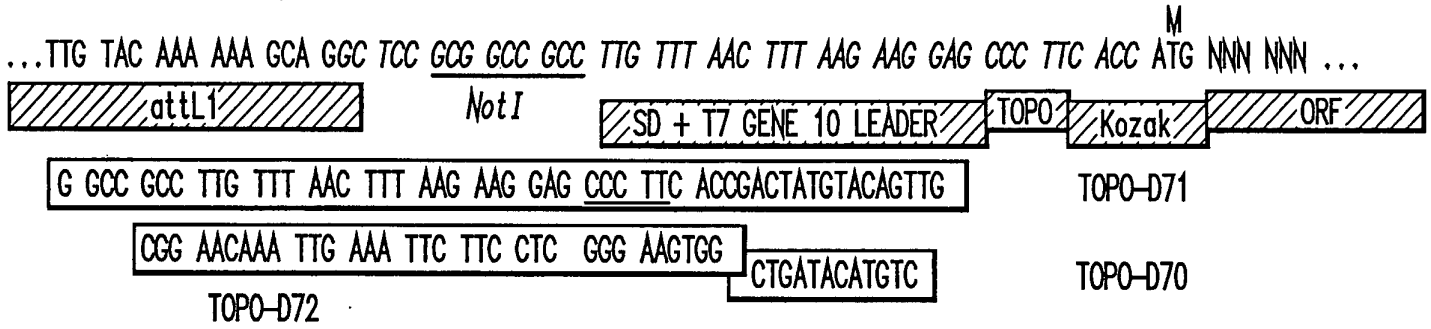
```
   1 gacggatcgg gagatctccc gatcccctat ggtgcactct cagtacaatc tgctctgatg
  61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
 121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
 181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
 241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
 301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
 361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
 421 attgacgtca atgggtggag tatttacggt aaactgccca cttggcagta catcaagtgt
 481 atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt
 541 atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca
 601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
 661 actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc
 721 aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatgggcg
 781 gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggctaact agagaaccca
 841 ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagt
 901 taagctatca acaagtttgt acaaaaaagc aggctccgcg gccgcccctt caccatgnnn
 961 nnnnnnaagg gtgggcgcgc cgacccagct ttcttgtaca aagtggttga tctagagggc
1021 ccgcggttcg aaggtaagcc tatccctaac cctctcctcg gtctcgattc tacgcgtacc
1081 ggttagtaat gagtttaaac gggggaggct aactgaaaca cggaaggaga caataccgga
1141 aggaacccgc gctatgacgg caataaaaag acagaataaa acgcacgggt gttgggtcgt
1201 ttgttcataa acgcggggtt cggtcccagg gctggcactc tgtcgatacc ccaccgagac
1261 cccattgggg ccaatacgcc cgcgtttctt ccttttcccc accccacccc ccaagttcgg
1321 gtgaaggccc agggctcgca gccaacgtcg gggcggcagg ccctgccata gcagatctgc
1381 gcagctgggg ctctaggggg tatccccacg cgccctgtag cggcgcatta agcgcggcgg
1441 gtgtggtggt tacgcgcagc gtgaccgcta cacttgccag cgccctagcg cccgctcctt
1501 tcgctttctt cccttccttt ctcgccacgt tcgccggctt tccccgtcaa gctctaaatc
1561 ggggcatccc tttagggttc cgatttagtg ctttacggca cctcgacccc aaaaaacttg
1621 attagggtga tggttcacgt agtgggccat cgccctgata gacggttttt cgccctttga
1681 cgttggagtc cacgttcttt aatagtggac tcttgttcca aactggaaca acactcaacc
1741 ctatctcggt ctattctttt gatttataag ggattttggg gatttcggcc tattggttaa
1801 aaaatgagct gatttaacaa aaatttaacg cgaattaatt ctgtggaatg tgtgtcagtt
1861 agggtgtgga aagtccccag gctccccagc aggcagaagt atgcaaagca tgcatctcaa
1921 ttagtcagca accaggtgtg gaaagtcccc aggctcccca gcaggcagaa gtatgcaaag
1981 catgcatctc aattagtcag caaccatagt cccgccccta actccgccca tcccgccccc
2041 aactccgccc agttccgccc attctccgcc ccatggctga ctaatttttt ttatttatgc
2101 agaggccgag gccgcctctg cctctgagct attccagaag tagtgaggag gctttttgg
2161 aggcctaggc ttttgcaaaa agctcccggg agcttgtata tccattttcg gatctgatca
2221 gcacgtgttg acaattaatc atcggcatag tatatcggca tagtataata cgacaaggtg
2281 aggaactaaa ccatggccaa gcctttgtct caagaagaat ccaccctcat tgaaagagca
2341 acggctacaa tcaacagcat ccccatctct gaagactaca gcgtcgccag cgcagctctc
2401 tctagcgacg gccgcatctt cactggtgtc aatgtatatc attttactgg gggaccttgt
2461 gcagaactcg tggtgctggg cactgctgct gctgcggcag ctggcaacct gacttgtatc
2521 gtcgcgatcg gaaatgagaa caggggcatc ttgagcccct gcggacggtg ccgacaggtg
2581 cttctcgatc tgcatcctgg gatcaaagcc atagtgaagg acagtgatgg acagccgacg
2641 gcagttggga ttcgtgaatt gctgccctct ggttatgtgt gggagggcta agcacttcgt
2701 ggccgaggag caggactgac acgtgctacg agatttcgat tccaccgccg ccttctatga
2761 aaggttgggc ttcggaatcg ttttccggga cgccggctgg atgatcctcc agcgcgggga-
```

FIG.25B

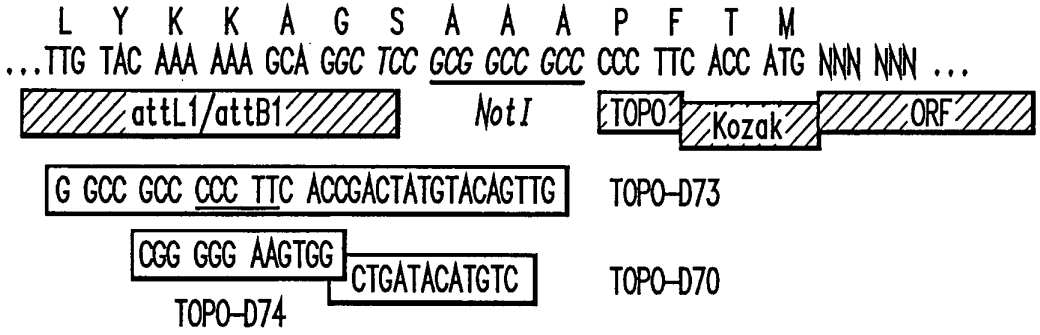
2821 tctcatgctg gagttcttcg cccaccccaa cttgtttatt gcagcttata atggttacaa
2881 ataaagcaat agcatcacaa atttcacaaa taaagcattt ttttcactgc attctagttg
2941 tggtttgtcc aaactcatca atgtatctta tcatgtctgt ataccgtcga cctctagcta
3001 gagcttggcg taatcatggt catagctgtt tcctgtgtga aattgttatc cgctcacaat
3061 tccacacaac atacgagccg gaagcataaa gtgtaaagcc tggggtgcct aatgagtgag
3121 ctaactcaca ttaattgcgt tgcgctcact gcccgctttc cagtcgggaa acctgtcgtg
3181 ccagctgcat taatgaatcg gccaacgcgc ggggagaggc ggtttgcgta ttgggcgctc
3241 ttccgcttcc tcgctcactg actcgctgcg ctcggtcgtt cggctgcggc gagcggtatc
3301 agctcactca aaggcggtaa tacggttatc cacagaatca ggggataacg caggaaagaa
3361 catgtgagca aaaggccagc aaaaggccag gaaccgtaaa aaggccgcgt tgctggcgtt
3421 tttccatagg ctccgccccc ctgacgagca tcacaaaaat cgacgctcaa gtcagaggtg
3481 gcgaaacccg acaggactat aaagatacca ggcgtttccc cctggaagct ccctcgtgcg
3541 ctctcctgtt ccgaccctgc cgcttaccgg atacctgtcc gcctttctcc cttcgggaag
3601 cgtggcgctt tctcatagct cacgctgtag gtatctcagt tcggtgtagg tcgttcgctc
3661 caagctgggc tgtgtgcacg aaccccccgt tcagcccgac cgctgcgcct tatccggtaa
3721 ctatcgtctt gagtccaacc cggtaagaca cgacttatcg ccactggcag cagccactgg
3781 taacaggatt agcagagcga ggtatgtagg cggtgctaca gagttcttga agtggtggcc
3841 taactacggc tacactagaa gaacagtatt tggtatctgc gctctgctga agccagttac
3901 cttcggaaaa agagttggta gctcttgatc cggcaaacaa accaccgctg gtagcggttt
3961 ttttgtttgc aagcagcaga ttacgcgcag aaaaaaagga tctcaagaag atcctttgat
4021 cttttctacg gggtctgacg ctcagtggaa cgaaaactca cgttaaggga ttttggtcat
4081 gagattatca aaaaggatct tcacctagat ccttttaaat taaaaatgaa gttttaaatc
4141 aatctaaagt atatatgagt aaacttggtc tgacagttac caatgcttaa tcagtgaggc
4201 acctatctca gcgatctgtc tatttcgttc atccatagtt gcctgactcc ccgtcgtgta
4261 gataactacg atacgggagg gcttaccatc tggccccagt gctgcaatga taccgcgaga
4321 cccacgctca ccggctccag atttatcagc aataaaccag ccagccggaa gggccgagcg
4381 cagaagtggt cctgcaactt tatccgcctc catccagtct attaattgtt gccgggaagc
4441 tagagtaagt agttcgccag ttaatagttt gcgcaacgtt gttgccattg ctacaggcat
4501 cgtggtgtca cgctcgtcgt ttggtatggc ttcattcagc tccggttccc aacgatcaag
4561 gcgagttaca tgatccccca tgttgtgcaa aaaagcggtt agctccttcg gtcctccgat
4621 cgttgtcaga agtaagttgg ccgcagtgtt atcactcatg gttatggcag cactgcataa
4681 ttctcttact gtcatgccat ccgtaagatg cttttctgtg actggtgagt actcaaccaa
4741 gtcattctga gaatagtgta tgcggcgacc gagttgctct tgcccggcgt caatacggga
4801 taataccgcg ccacatagca gaactttaaa agtgctcatc attggaaaac gttcttcggg
4861 gcgaaaactc tcaaggatct taccgctgtt gagatccagt tcgatgtaac ccactcgtgc
4921 acccaactga tcttcagcat cttttacttt caccagcgtt tctgggtgag caaaaacagg
4981 aaggcaaaat gccgcaaaaa agggaataag ggcgacacgg aaatgttgaa tactcatact
5041 cttccttttt caatattatt gaagcattta tcagggttat tgtctcatga gcggatacat
5101 atttgaatgt atttagaaaa ataaacaaat aggggttccg cgcacatttc cccgaaaagt
5161 gccacctgac gtc

FIG.25C

pENTR/SD–dTopo: 5' END



pENTR–dTOPO AND pcDNAGW–dTOPO: 5' END



pENTR/SD–dTOPO, pENTR–dTOPO, AND pcDNAGW–dTOPO: 3' END

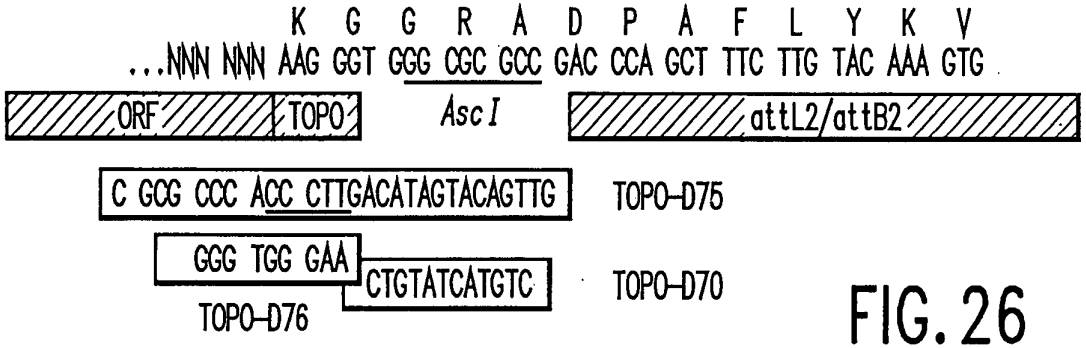


FIG. 26

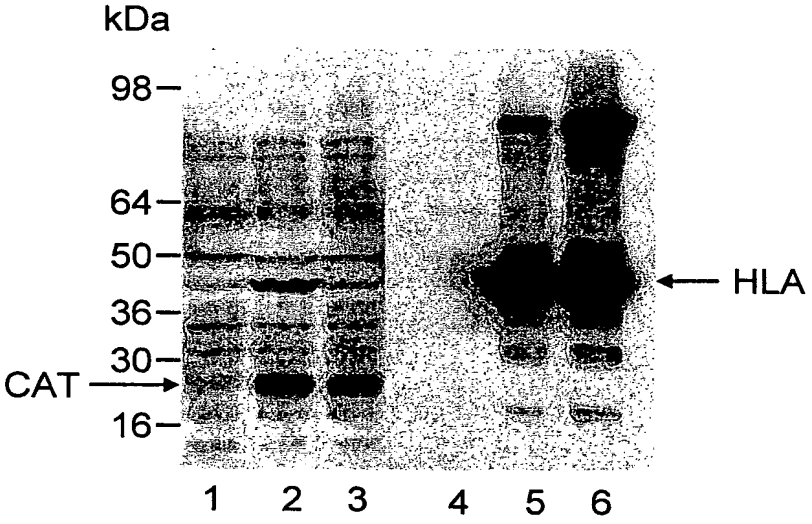


FIG.27

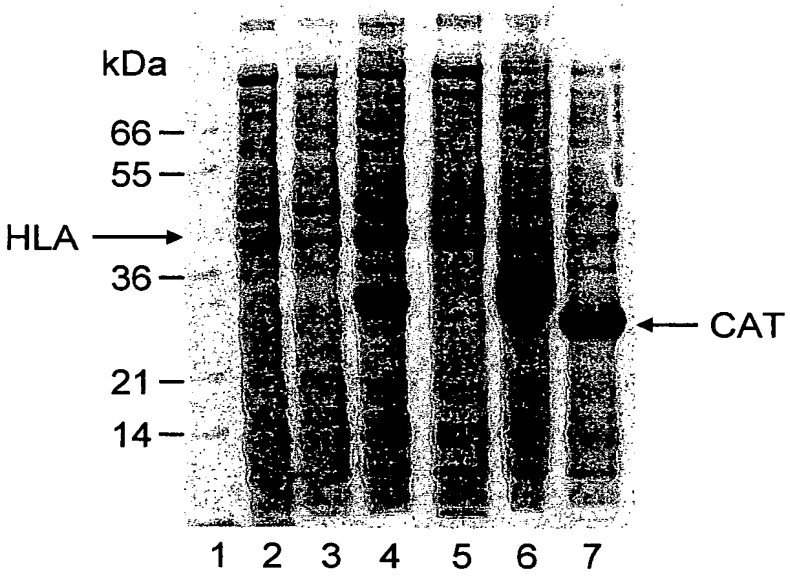


FIG.28

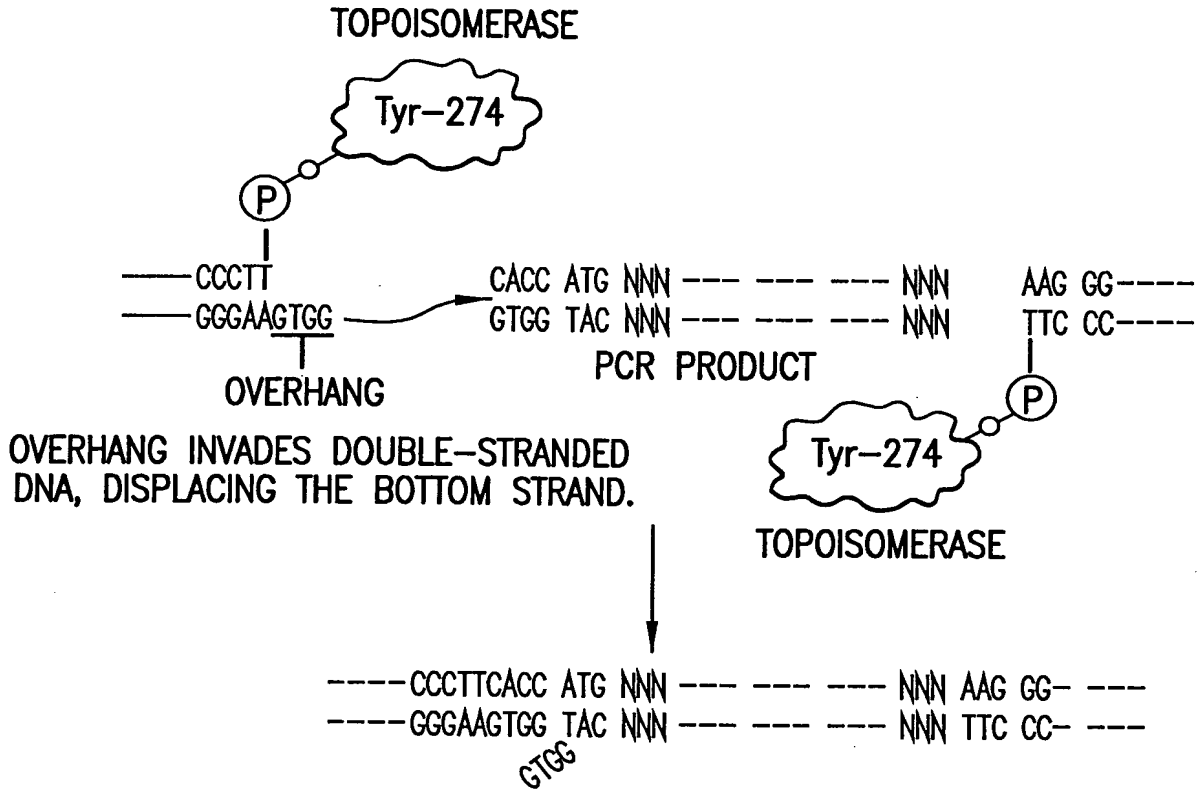
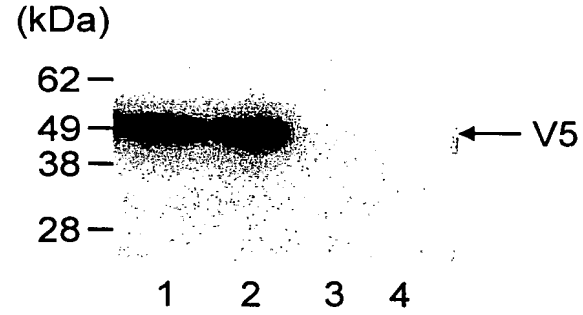
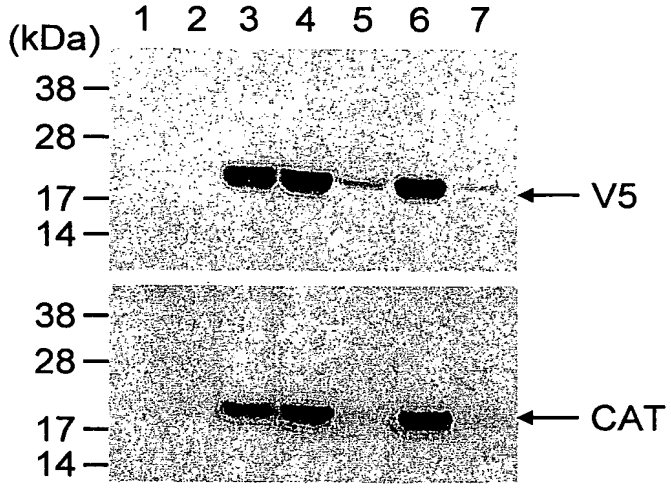

TOPOISOMERASE
Tyr–274
P
CCCTT
GGGAAGTGG
OVERHANG
CACC ATG NNN --- --- --- NNN
GTGG TAC NNN --- --- --- NNN
AAG GG----
TTC CC----
PCR PRODUCT
P
Tyr–274
TOPOISOMERASE
OVERHANG INVADES DOUBLE–STRANDED DNA, DISPLACING THE BOTTOM STRAND.
----CCCTTCACC ATG NNN --- --- --- NNN AAG GG- ---
----GGGAAGTGG TAC NNN --- --- --- NNN TTC CC- ---
GTGG

FIG. 29



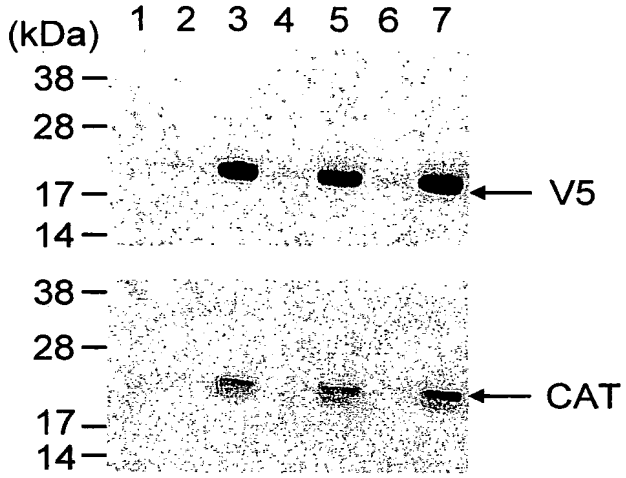
Lane 1: pCMVTetO/CAT/V5TKpA (without secondary PCR) + Tet
Lane 2: pCMVTetO/CAT/V5TKpA (with secondary PCR) + Tet
Lane 3: pCMVTetO/CAT/V5TKpA (with secondary PCR) - Tet
Lane 4: pCMVTetO/CAT/V5TKpA (without secondary PCR) - Tet

FIG.30A



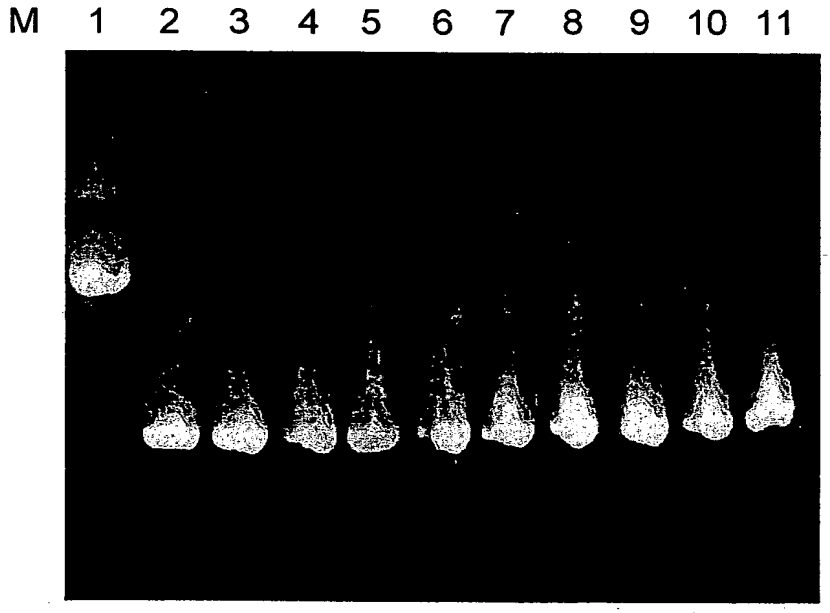
Lane 1: TRex-CHO Cells + Tet
Lane 2: without secondary PCR (with purified CAT) - Tet
Lane 3: without secondary PCR (with purified CAT) + Tet
Lane 4: without secondary PCR (with unpurified CAT) + Tet
Lane 5: without secondary PCR (with unpurified CAT) -Tet
Lane 6: with secondary PCR + Tet
Lane 7: with secondary PCR - Tet

FIG.30B



Lane 1: TRex-293 Cells + Tet
Lane 2: without secondary PCR (with purified CAT) - Tet
Lane 3: without secondary PCR (with purified CAT) + Tet
Lane 4: without secondary PCR (with unpurified CAT) - Tet
Lane 5: without secondary PCR (with unpurified CAT) +Tet
Lane 6: with secondary PCR - Tet
Lane 7: with secondary PCR + Tet

FIG.30C



Lane 1: negative control; lanes 2-11: test clones; M: 500 bp marker

FIG.31

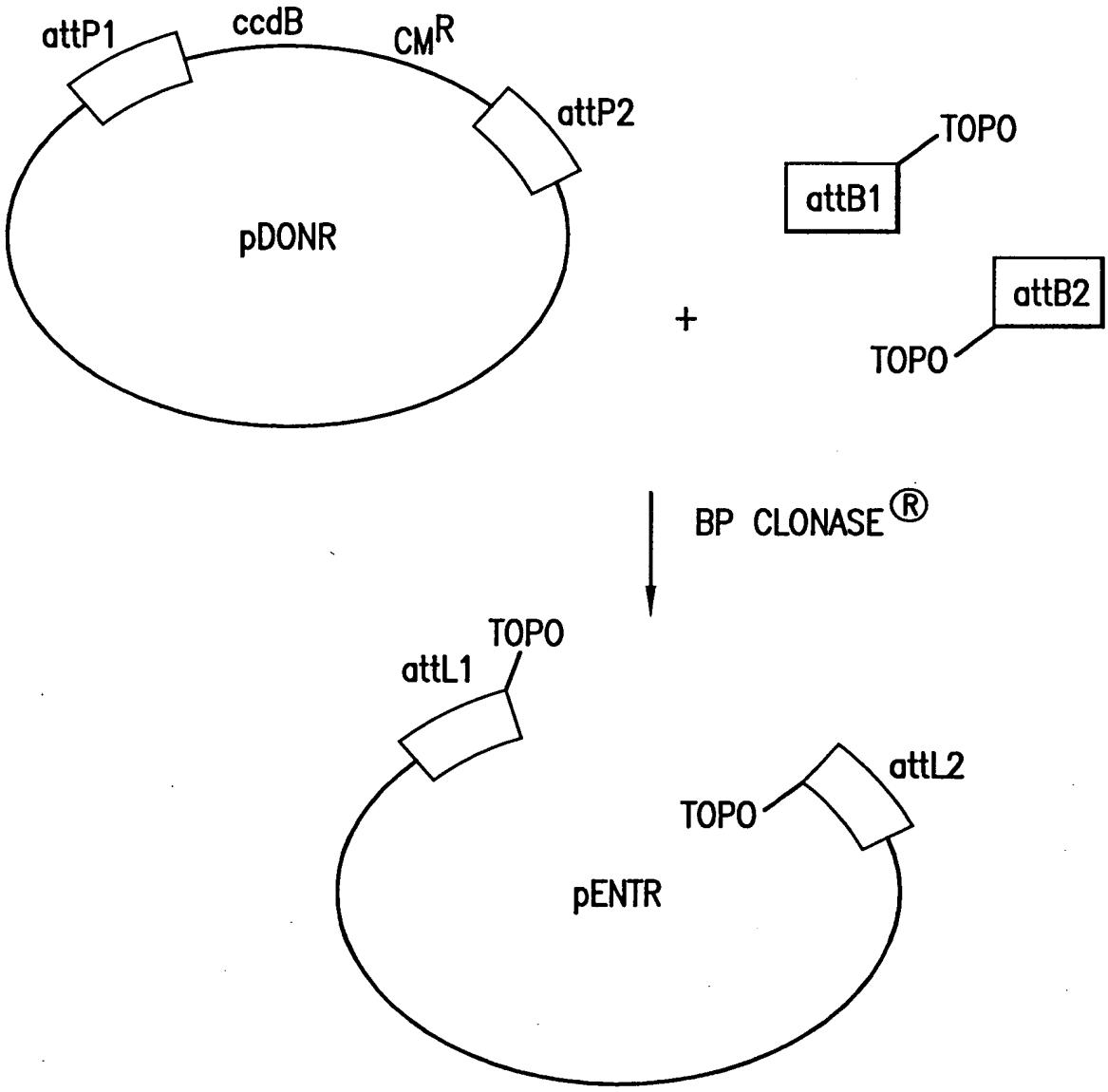

attP1
ccdB
CM^R
attP2
pDONR
TOPO
attB1
attB2
TOPO
+
BP CLONASE®
TOPO
attL1
attL2
TOPO
pENTR

FIG. 32

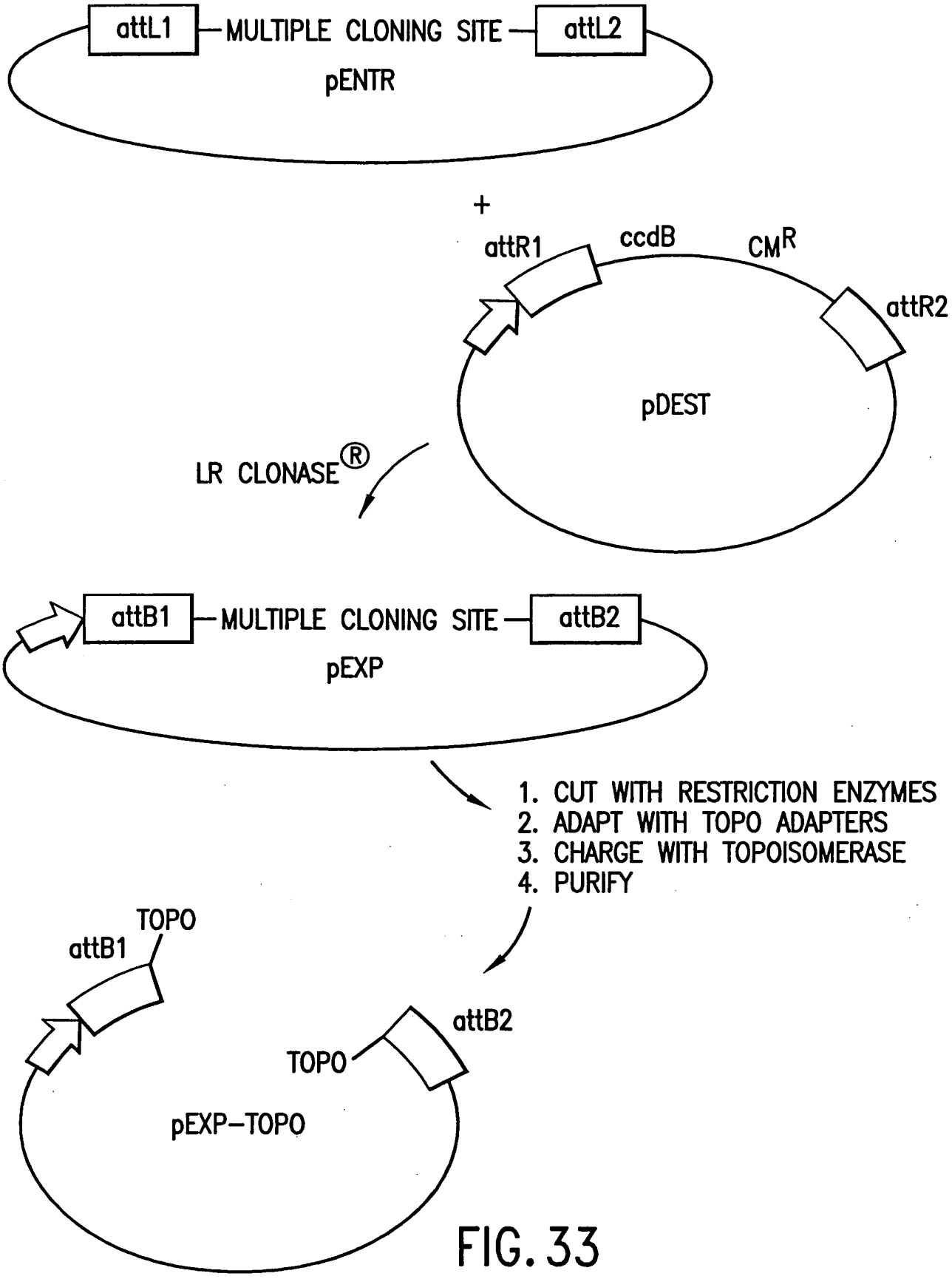

attL1
MULTIPLE CLONING SITE
attL2
pENTR
+
attR1
ccdB
CMR
attR2
pDEST
LR CLONASE®
attB1
MULTIPLE CLONING SITE
attB2
pEXP
1. CUT WITH RESTRICTION ENZYMES
2. ADAPT WITH TOPO ADAPTERS
3. CHARGE WITH TOPOISOMERASE
4. PURIFY
TOPO
attB1
attB2
TOPO
pEXP–TOPO

FIG. 33

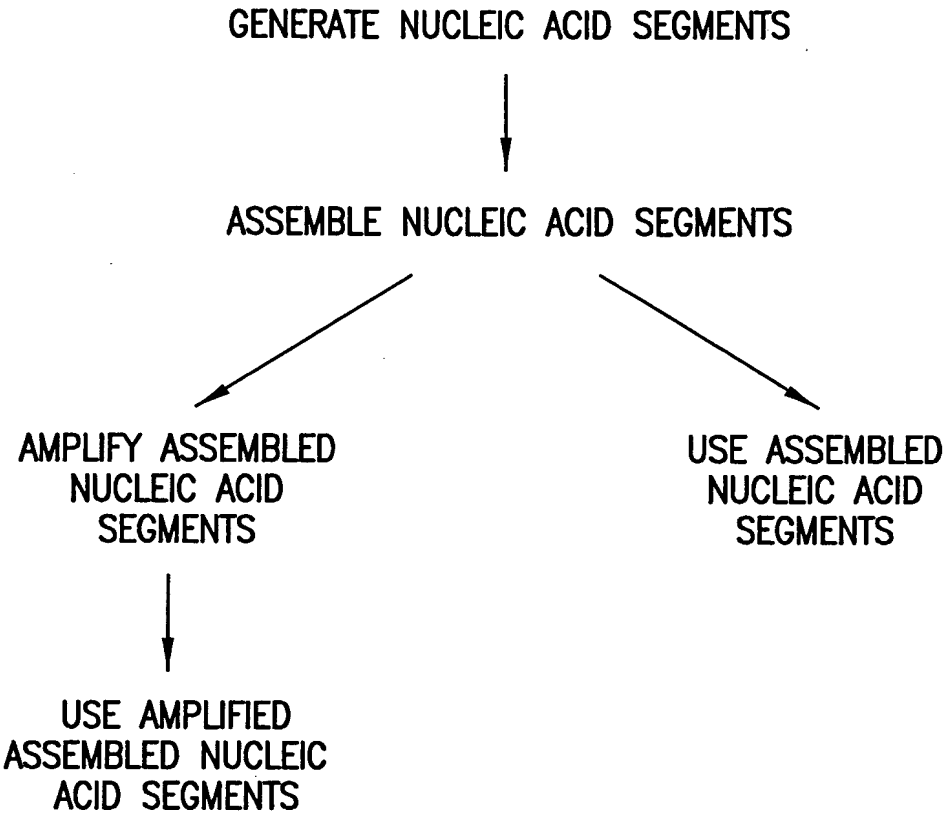

GENERATE NUCLEIC ACID SEGMENTS
ASSEMBLE NUCLEIC ACID SEGMENTS
AMPLIFY ASSEMBLED NUCLEIC ACID SEGMENTS
USE ASSEMBLED NUCLEIC ACID SEGMENTS
USE AMPLIFIED ASSEMBLED NUCLEIC ACID SEGMENTS

FIG. 34

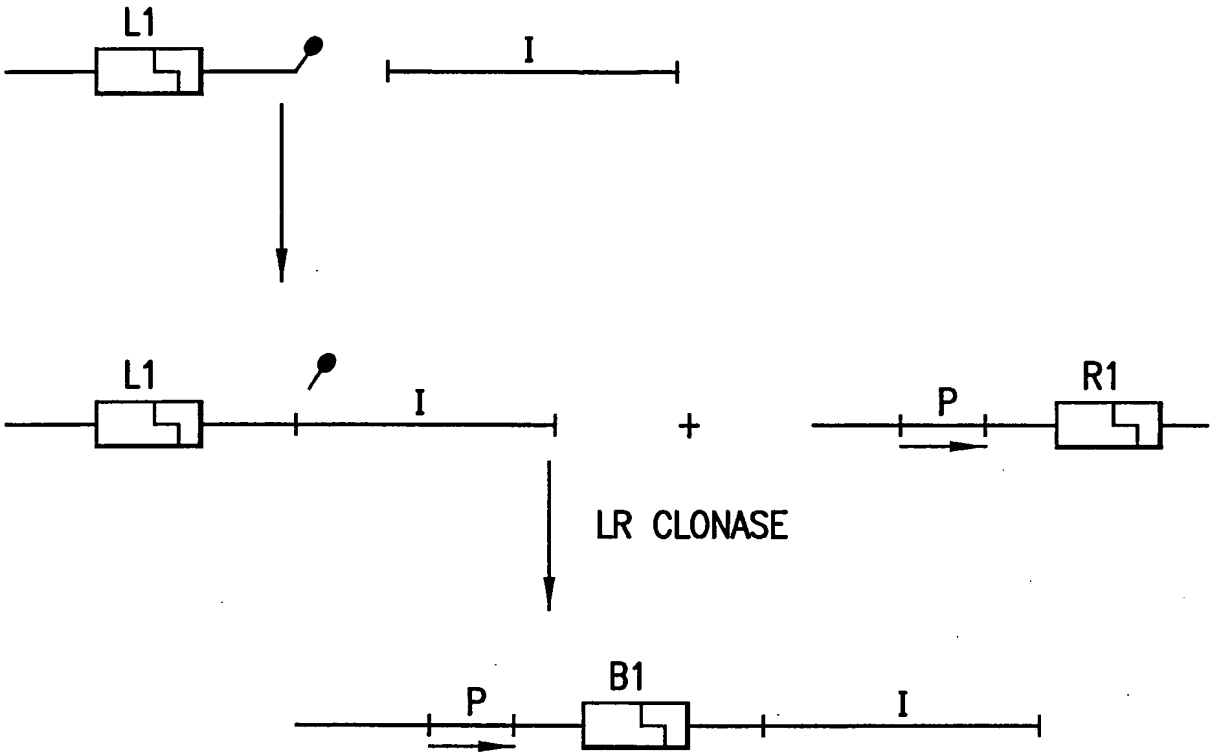


FIG. 35

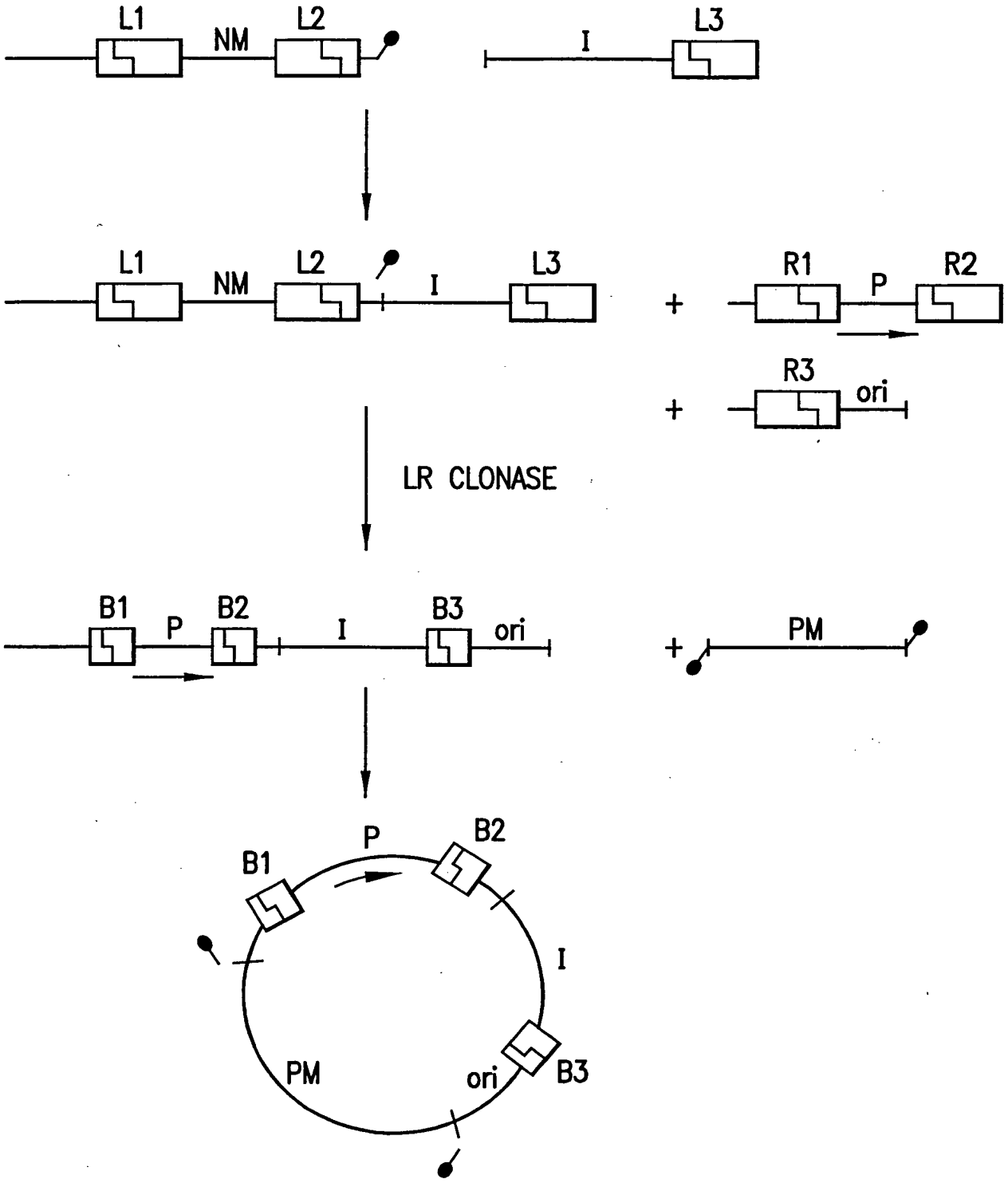

L1
NM
L2
I
L3
L1
NM
L2
I
L3
R1
P
R2
R3
ori
LR CLONASE
B1
P
B2
I
B3
ori
PM
P
B2
B1
I
PM
ori
B3

FIG. 36

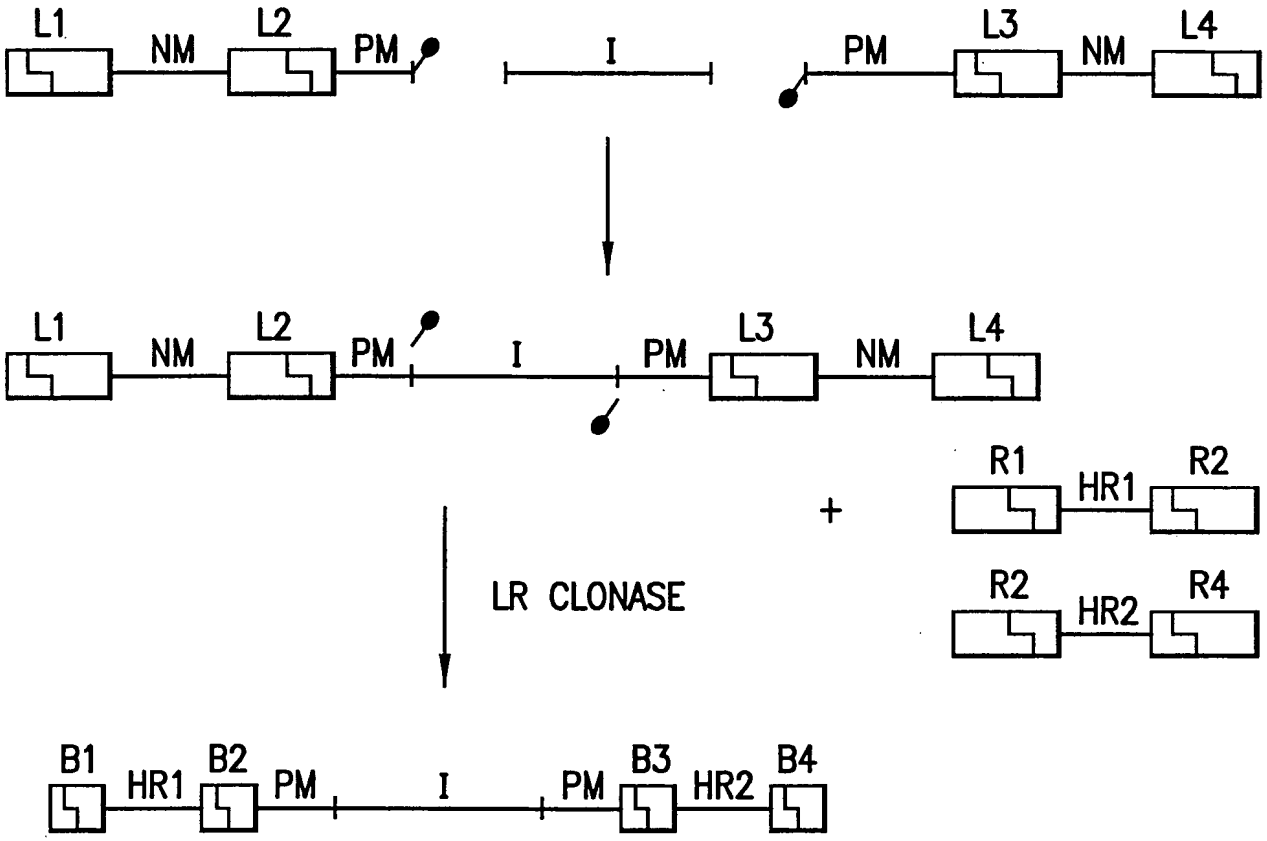

L1
NM
L2
PM
I
PM
L3
NM
L4
L1
NM
L2
PM
I
PM
L3
NM
L4
R1
HR1
R2
+
LR CLONASE
R2
HR2
R4
B1
HR1
B2
PM
I
PM
B3
HR2
B4

FIG. 37

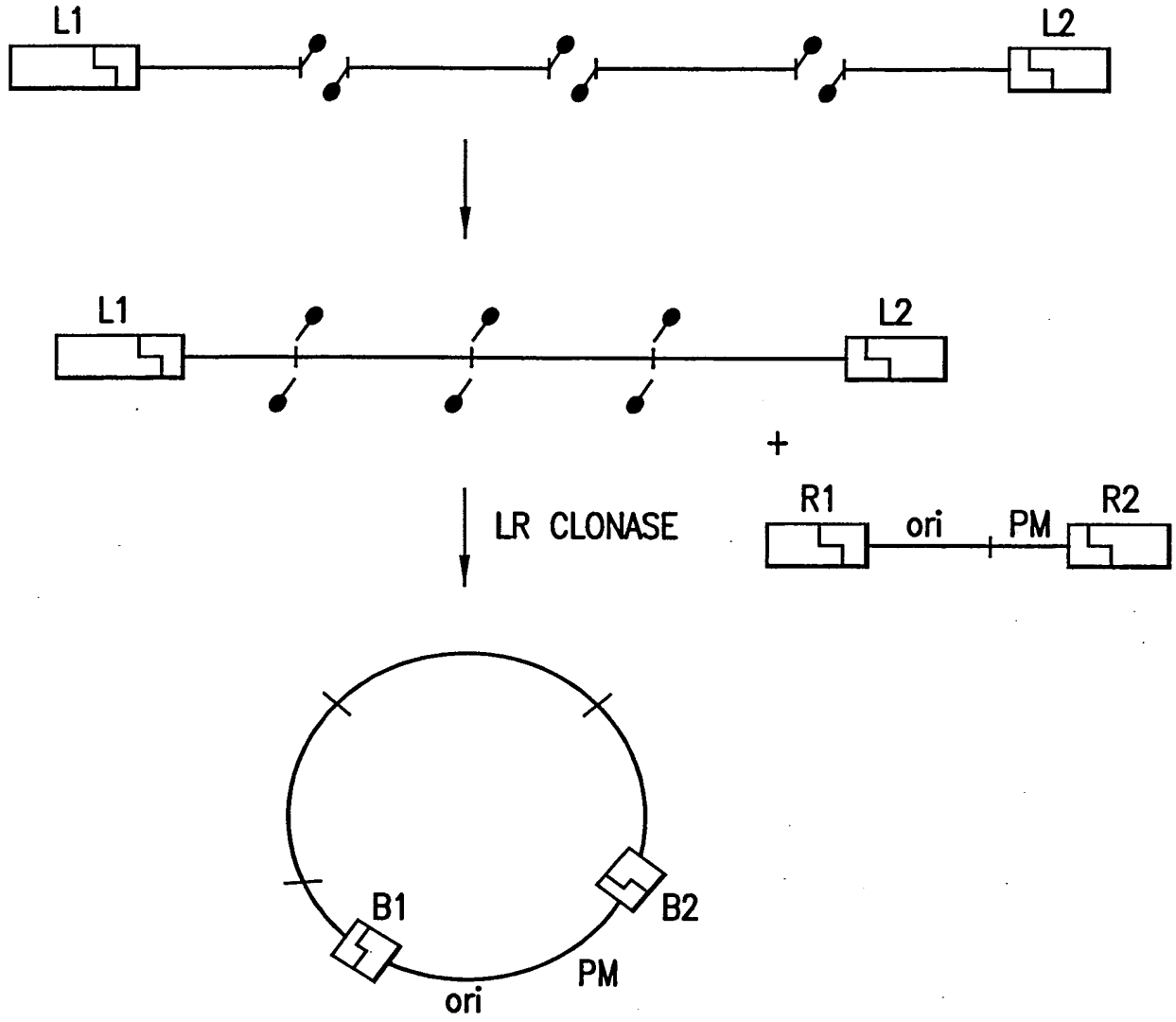


FIG. 38

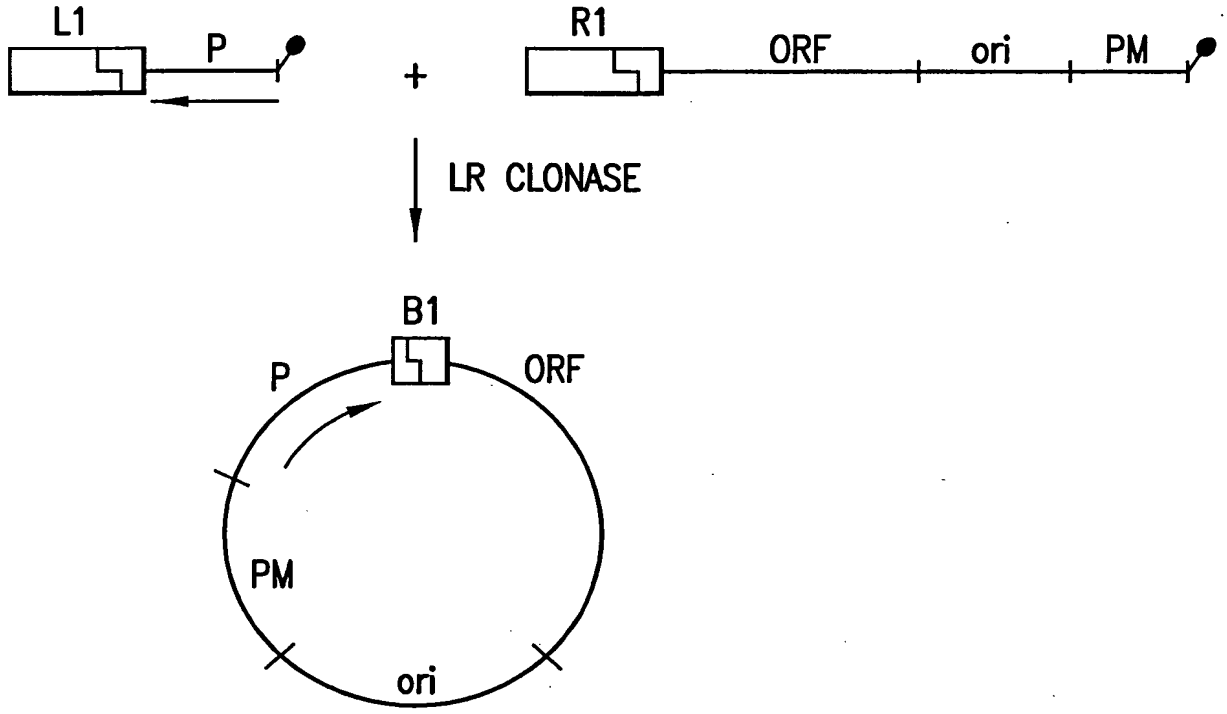


FIG. 39

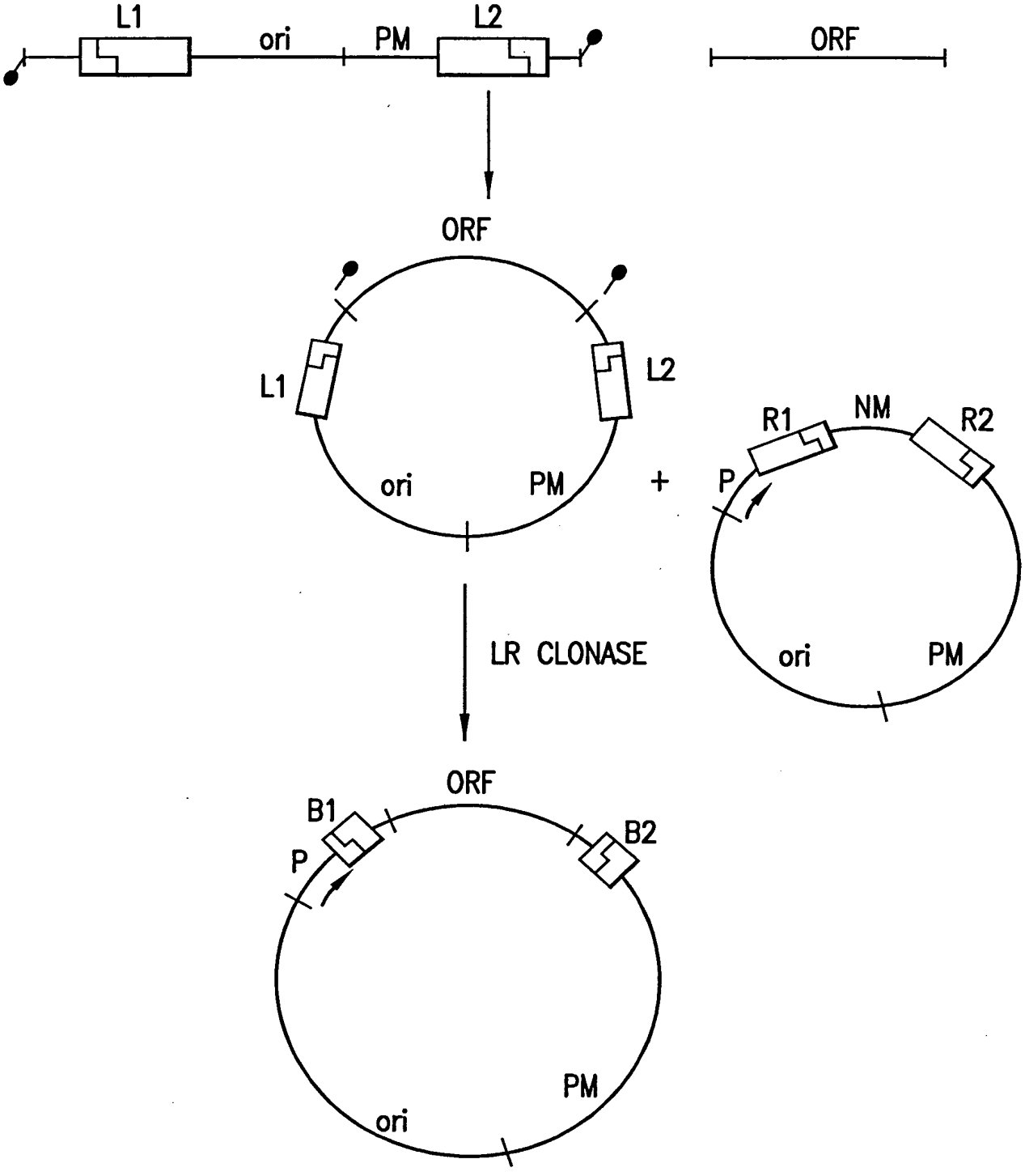

L1
ori
PM
L2
ORF
ORF
L1
L2
ori
PM
+
R1
NM
R2
P
ori
PM
LR CLONASE
ORF
B1
B2
P
PM
ori

FIG. 40

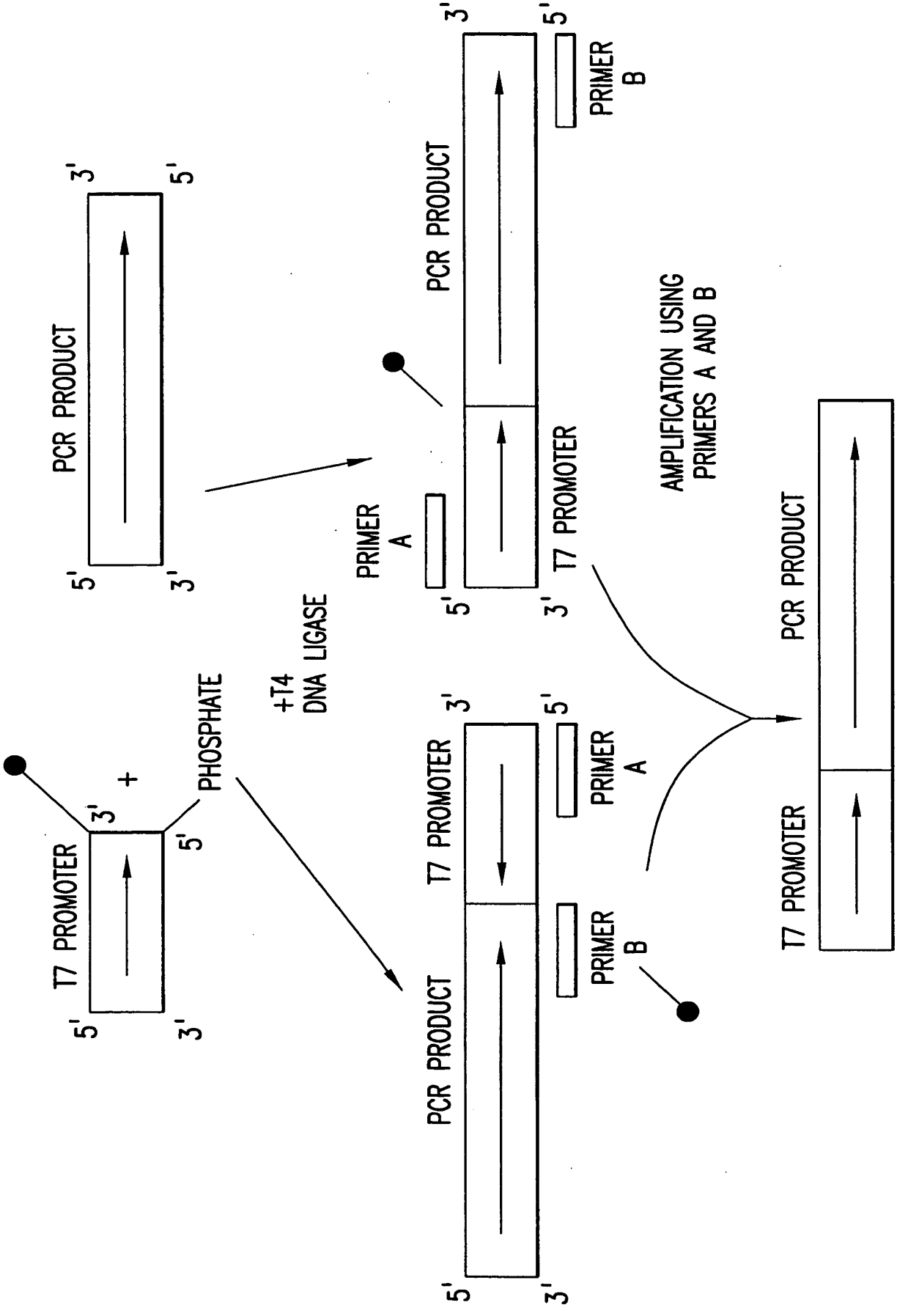

PCR PRODUCT
3'
5'
5'
3'
PRIMER A
PRIMER B
T7 PROMOTER
PHOSPHATE
+T4 DNA LIGASE
AMPLIFICATION USING PRIMERS A AND B

FIG.41

T7 promoter TOPO

5' pGACTCGTAATACGACTCACTATAGGGCCCTT 3'

3' AAAAAAAAAAAACTGAGCATTATGCTGAGTGATATCCCGGGAp 5'

FIG.42A

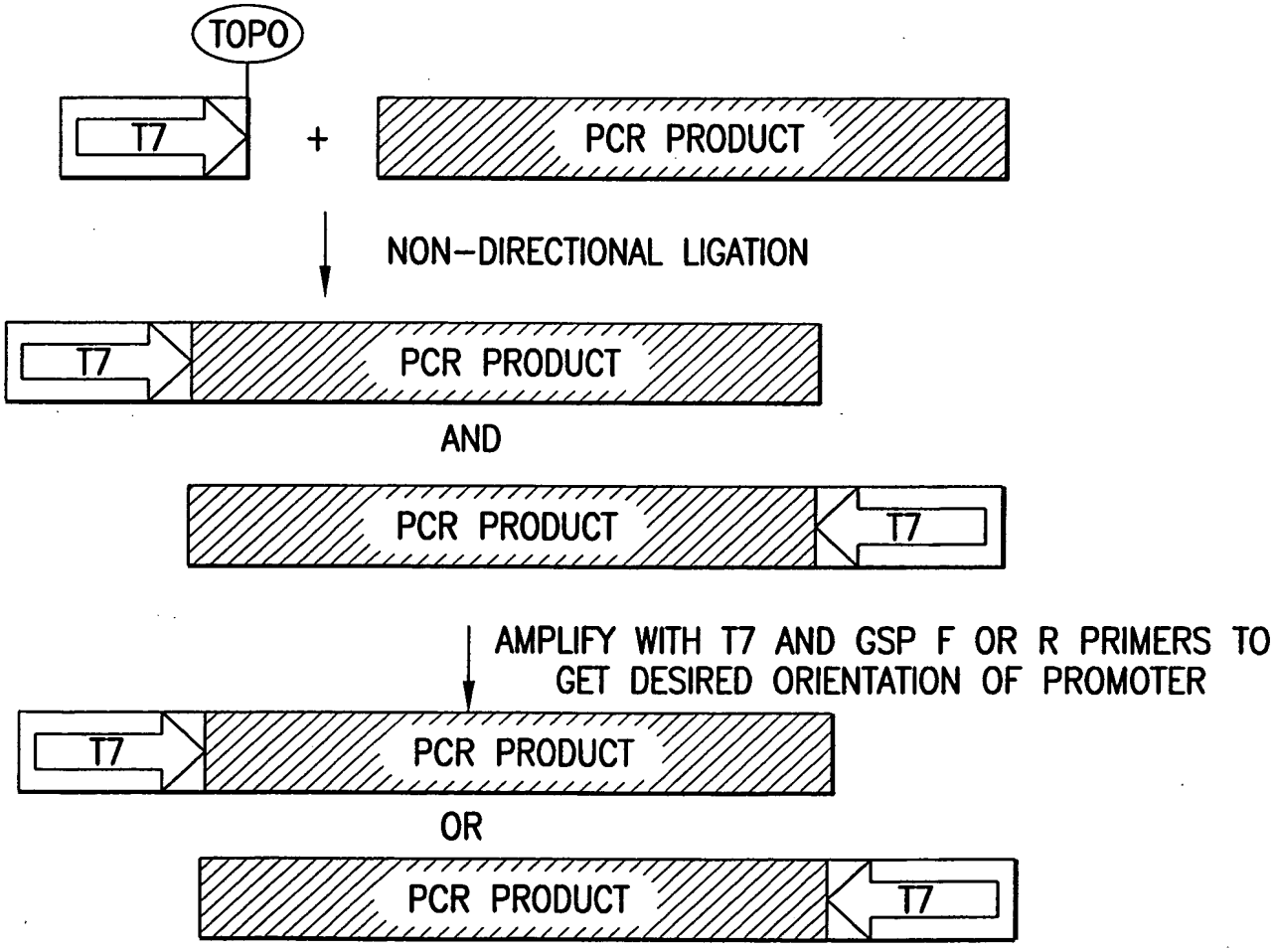


FIG.42B

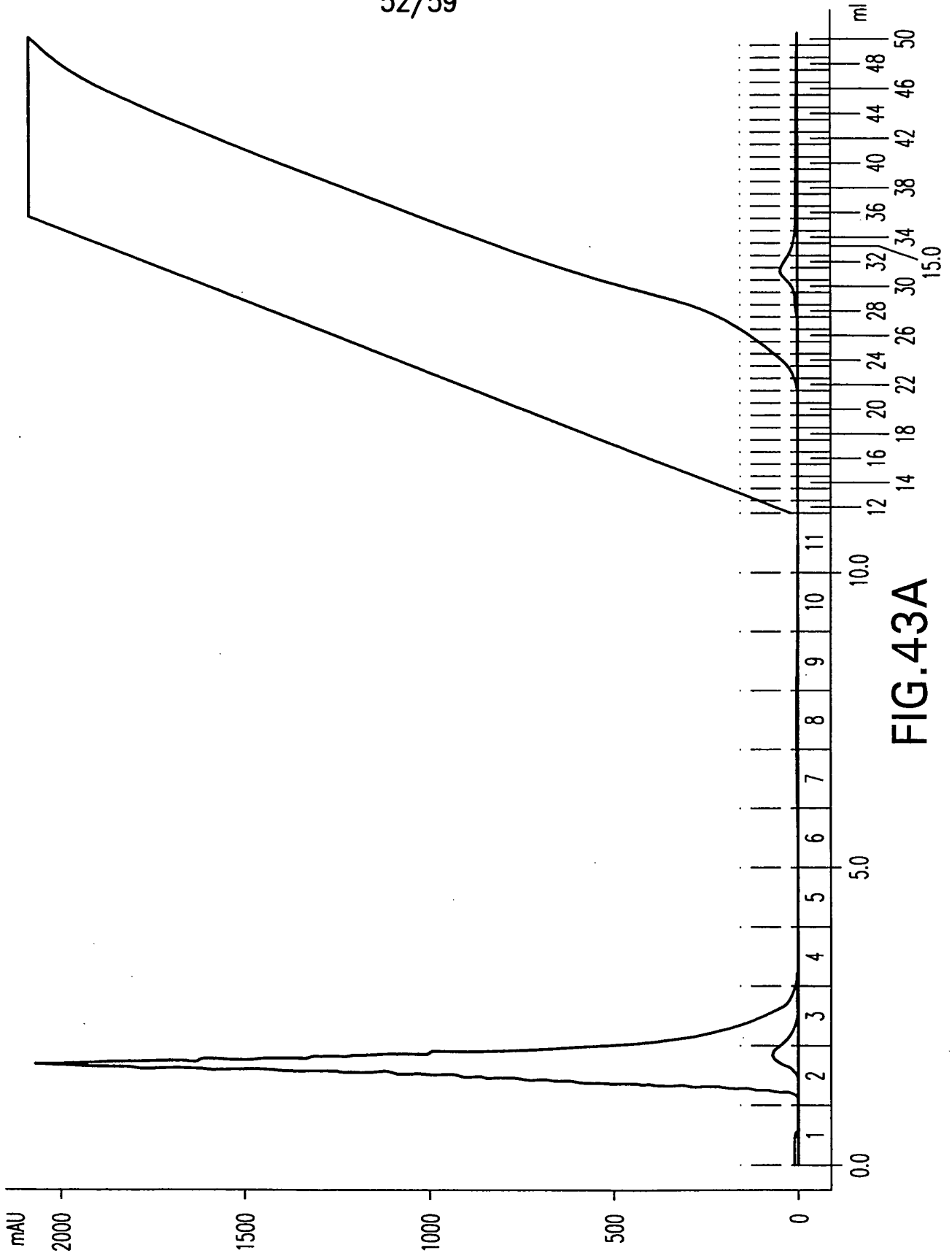

mAU
2000
1500
1000
500
0
0.0
5.0
10.0
15.0
ml
1
2
3
4
5
6
7
8
9
10
11
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50

FIG.43A

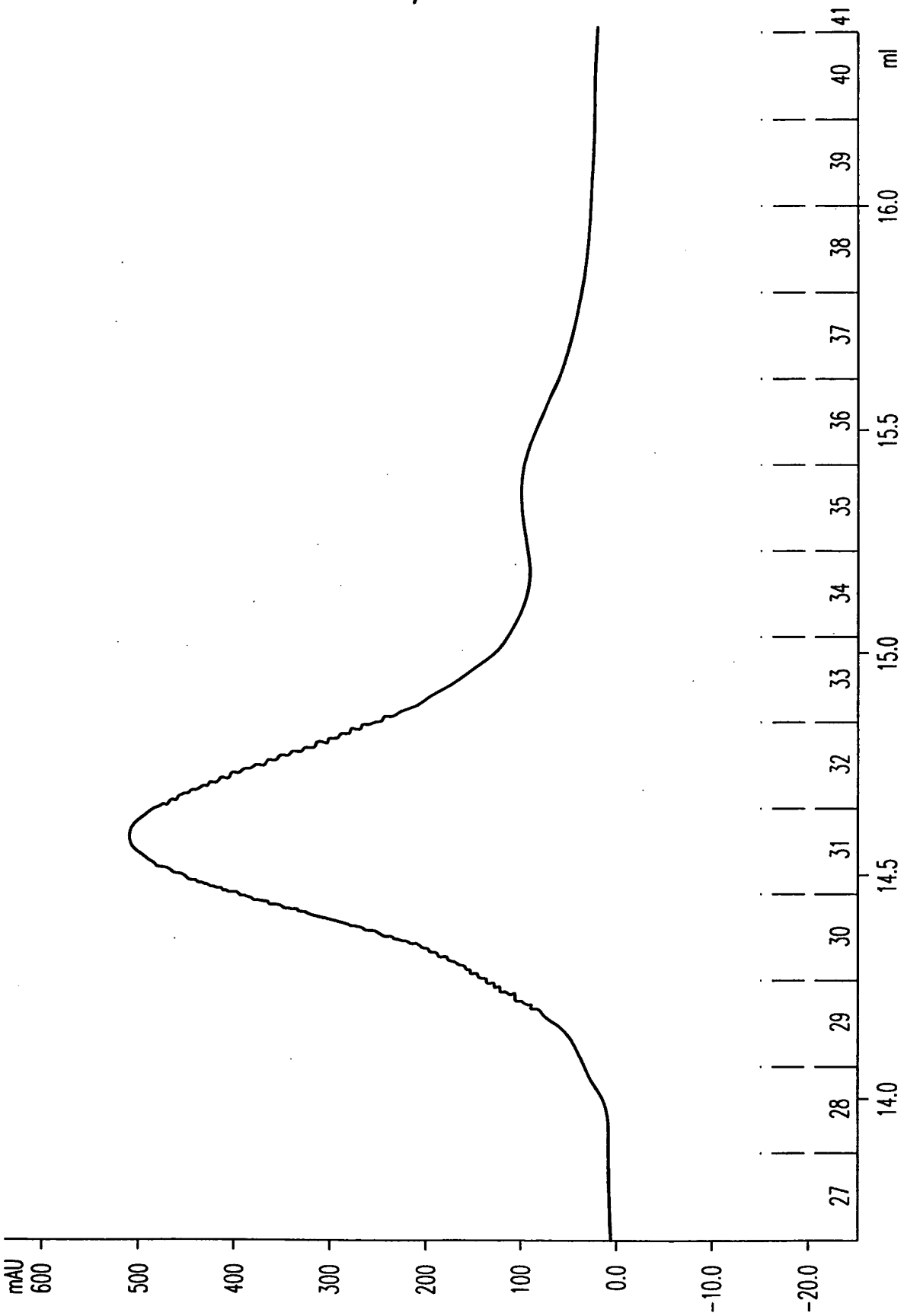

mAU
600
500
400
300
200
100
0.0
-10.0
-20.0
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
14.0
14.5
15.0
15.5
16.0
ml

FIG.43B

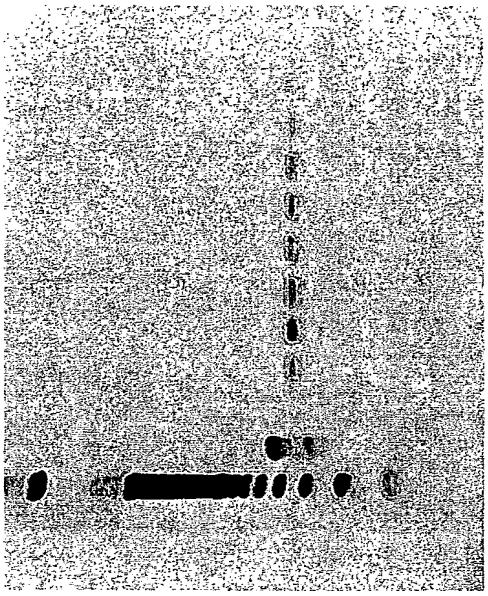

undigested load
annealed oligos
load
F-T2
F-T3
F-T4
F-T5
29
30
31
annealed oligos
32
33
34
35
36
37
38
39
40

FIG.44A

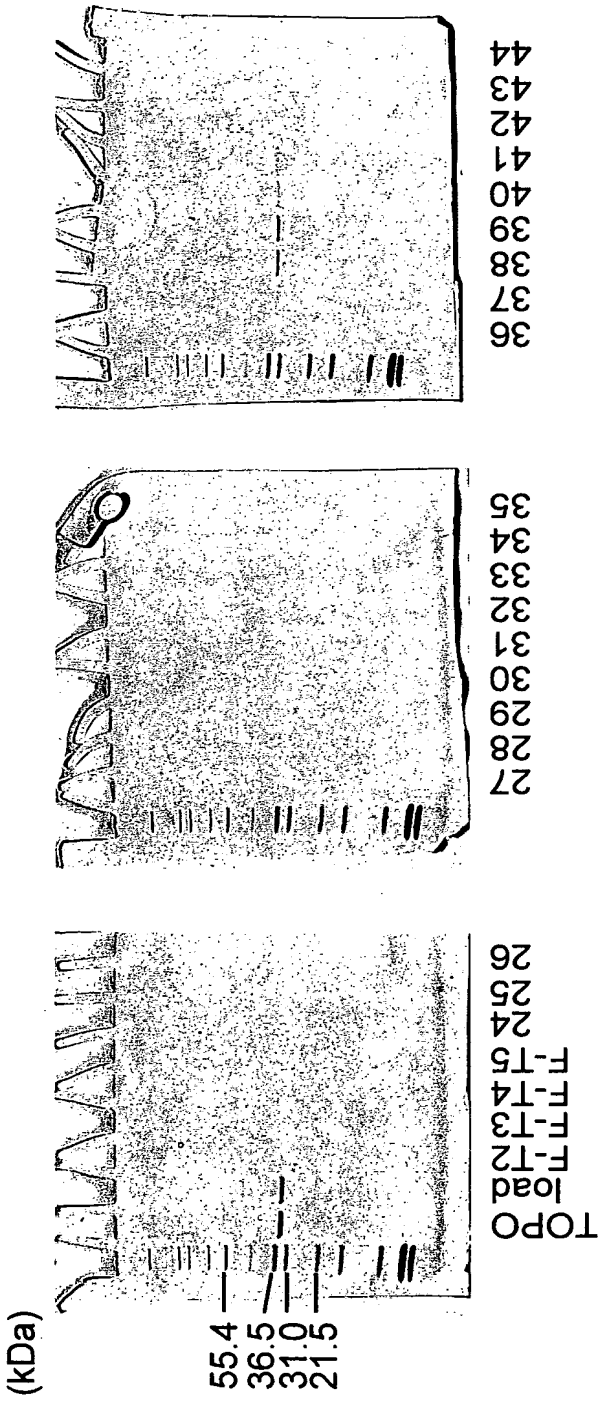

(kDa)
55.4
36.5
31.0
21.5
TOPO
load
F-T2
F-T3
F-T4
F-T5
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44

FIG.44B

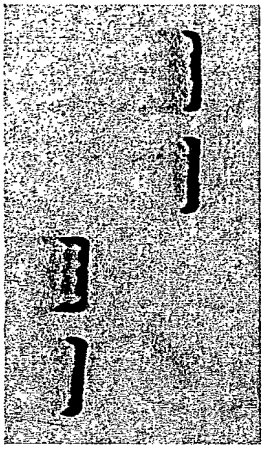


FIG.45B

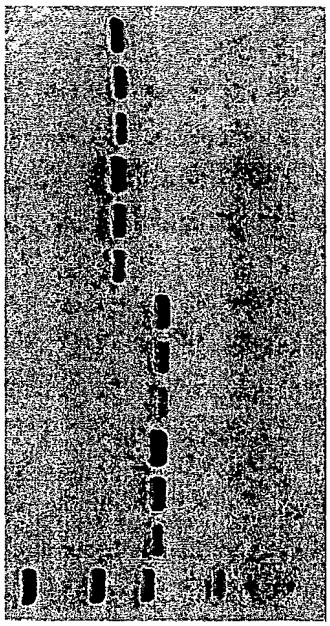


FIG.45A

57/59

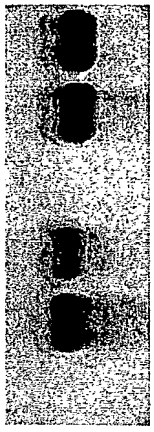


FIG.45D

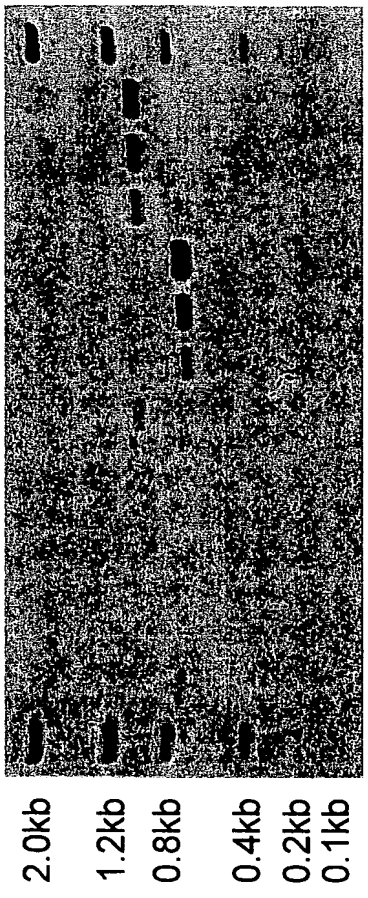


FIG.45C

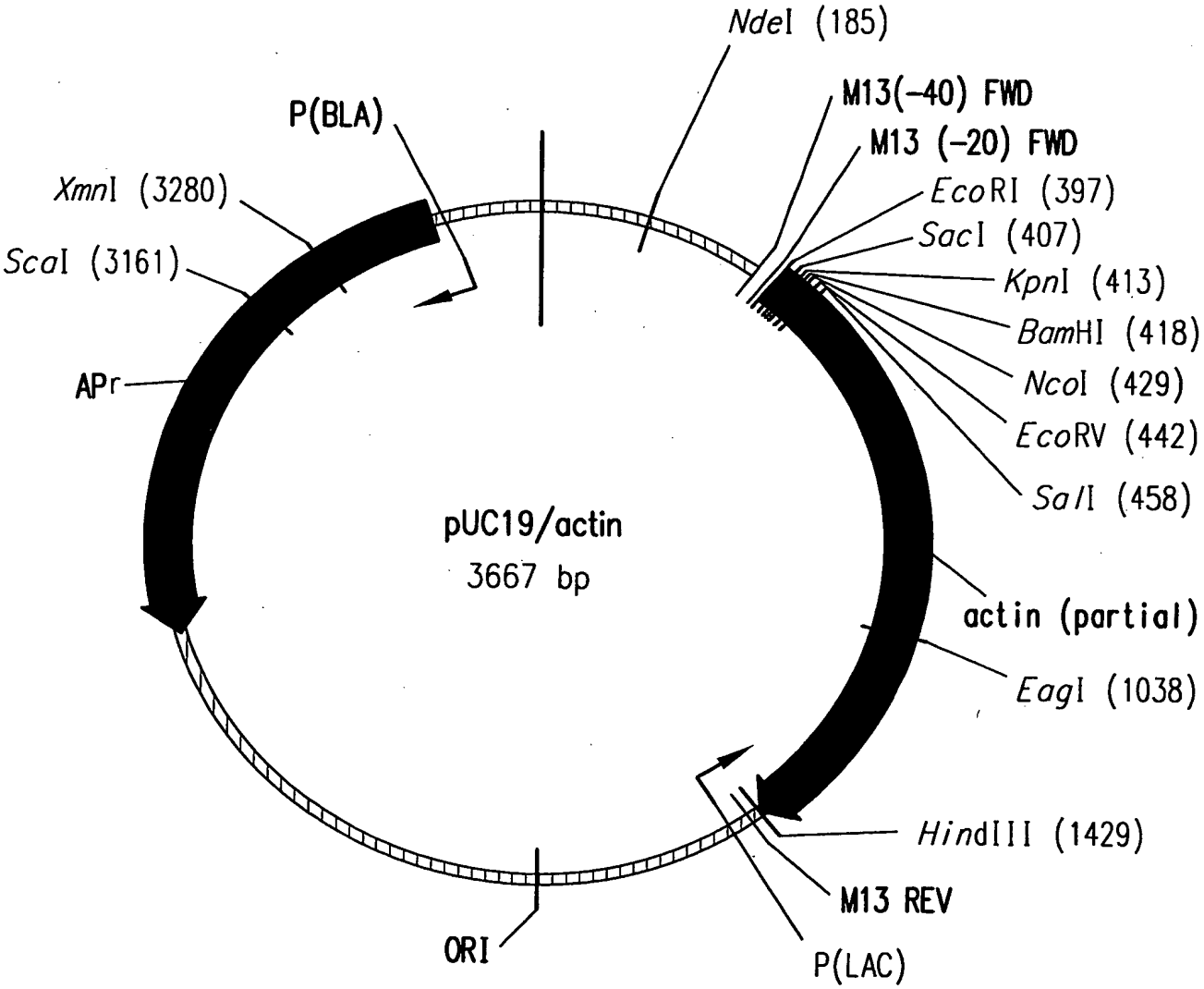
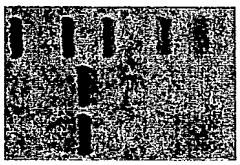

NdeI (185)
M13(-40) FWD
M13 (-20) FWD
P(BLA)
EcoRI (397)
XmnI (3280)
SacI (407)
ScaI (3161)
KpnI (413)
BamHI (418)
APr
NcoI (429)
EcoRV (442)
SalI (458)
pUC19/actin
3667 bp
actin (partial)
EagI (1038)
HindIII (1429)
M13 REV
ORI
P(LAC)

FIG.46A

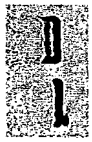
T7-actin 1° PCR transcription
actin 2° PCR transcription

FIG.46D



T7-actin 1° PCR
actin 2° PCR

FIG.46C



actin PCR + linker
actin 1° PCR mock linking

FIG.46B